Biochemical Characterization of Drosophila Insulin-like Peptides

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Summary

Members of the Insulin family of proteins and hormones have emerged as important players in regulating two of the fundamental processes of life, namely growth and metabolism. Insulin plays a pivotal role in the regulation of sugar levels in the blood. Administration of Insulin has been used to treat disease conditions like Diabetes mellitus where blood sugar levels are not regulated naturally, either due to lack of Insulin itself or due to peripheral body tissues developing resistance to Insulin stimulation. Insulin-like growth factors are structurally very similar to Insulin, i.e. they contain the Insulin hallmark- six cysteines forming disulphide bonds. Two IGFs have been characterized in mammals which play a very important role in regulating pre- and post- natal growth. Both Insulin and IGFs are hormonal ligands which bind to an Insulin receptor on the surface of the cells, which leads to conformational changes and phosphorylation of the receptor. The 'activated' receptor in turn activates/changes phosphorylation state of several membrane-bound, cytoplasmic and nuclear proteins, hence forming a signaling cascade. The type of downstream proteins activated by the receptor varies with cell or tissue type, thus leading to differential response tissues to the same signal. Recently more Insulin-like ligands have been identified in mice and humans. However the receptors to which they bind to or their functions have not been fully characterized. Recently, the receptor for Relaxin, a member of the insulin family, was identified to be a G-protein coupled receptor.

Much of the knowledge on insulin-like molecules and their functions comes from studies on mammalian model systems. Studies in these systems have added valuable knowledge to the understanding and treatment of major diseases like cancer and diabetes. Homologs of the insulin signaling pathway components have been shown to be conserved in lower organisms like *Caenorhabditis elegans* and *Drosophila melanogaster*. In fact, Drosophila has contributed significantly in identifying and defining functions of new components involved in insulin signaling. However, the signaling ligands have been recently identified in these model systems and are less well characterized in lower organisms. The study of insulin-like ligands in lower organisms might add valuable information to the existing knowledge from mammalian systems. In 2000, seven Insulin-like genes were identified in the Drosophila genome each of which codes for a putative insulin-like molecule, named Drosophila insulin-like peptides 1-7 (DILP 1-7). Study of the expression pattern of these genes showed expression of at least three *dilp* transcripts in particular brain neurons. Ablation of these neurons during development lead to smaller animals with higher sugar and lipid levels, suggesting that the peptides play a similar role in Drosophila as Insulin and IGFs do in

mammals. However, the functional and biochemical characterization of the Drosophila insulins was not complete. The aim of this Ph.D. project was to characterize the Drosophila Insulin-like peptides further.

To study and compare the growth regulating effects of DILPs they were overexpressed ubiquitously using the UAS-GAL4 system. Weak ubiquitous overexpression showed an increase in body weight of adult males and females, with DILP 2 showing the strongest growth promoting effect. To study and compare their binding and signaling abilities, it was imperative to purify the DILPs. DILP 2, 3, 4 and 5 were synthesized and purified by standard methods used for the purification of human insulin. The activities of the DILPs were tested and compared by assessing phosphorylation of two downstream kinases of the insulin signaling cascade, namely Protein kinase B and S6 kinase. DILP 2 proved to be most potent stimulator followed by DILP 3, DILP 4 and DILP 5. The binding affinity of DILP 2, as determined by competitive binding assay, was found to be higher than DILP 3, which might also explain the difference between activities of various DILPs. Flow cytometry and BrdU incorporation studies were performed to study the effect of individual DILPs on cell size and cell cycle progression. The results show that DILP stimulated cells were slightly larger and showed higher percentage of cells in S phase, as compared to unstimulated control cells. To study their role on stimulating cells to take up sugar, glucose uptake assays were performed on cells and tissues. Under the conditions tested, the cells and tissues did not show any increase in the uptake of glucose upon stimulation with any of the DILPs. This however does not rule out the possibility that the DILPs might be playing such a role in sugar homeostasis physiologically. The single Drosophila Insulin receptor shows variable abundance in various tissue types. Preliminary experiments show that various tissues get 'sensitized' to insulin stimulation under low nutrient conditions.

This study has helped in better understanding of the roles that the insulin-like peptides might play in *Drosophila melanogaster*.

Zusammenfassung

Die Mitglieder der Insulin Familie haben sich als sehr wichtige Regulatoren zweier grundsätzlicher Lebensprozesse herausgestellt - Wachstum und Metabolismus. Insulin spielt eine wichtige Rolle in der Regulation des Blutzuckerspiegels. Insulin wurde hauptsächlich verabreicht zur Behandlung von Krankheiten wie Diabetes mellitus bei welcher die Höhe des Blutzuckerspiegels nicht natürlich reguliert werden kann, da entweder die Insulinmenge nicht ausreicht oder die periphären Gewebe eine Resistanz gegen die Insulin Stimulation aufweisen. Insulin-like growth factors sind strukturell sehr ähnlich mit Insulin, unter anderem enthalten sie die Insulin Hauptstruktur - die Disulphidbrückenbildenden 6 Cysteine. Zwei IGFs, die eine sehr wichtige Rolle in prä- und postnatalem Wachstum spielen, sind in Säugetieren characterisiert worden. Insulin und IGFs sind hormonartige Liganden, die an einem Insulin Rezeptor auf der Zelloberfläche binden und dadurch zu einer Konformationsänderung und Phosphorylierung des Rezeptors anführen. Die Aktivierung des Rezeptors führt zu einer Änderung des PhosphorylierungsStatus mehrerer membranbindenden, Zytoplasmatischen und Kern lokalisierten Proteinen. Die Art der nachgeschalteten Proteine, die vom Rezeptor aktiviert werden, ist in verschiedenen Gewebetypen unterschiedlich, so dass das selbe Signal in verschiedenen Geweben zu anderen Signalantworten führen kann. Erst kürzlich sind einige neue Insulin-like Liganden in Mäusen und dem Menschen entdeckt worden, deren Rezeptoren als auch deren Funktion sind jedoch noch nicht vollständig characterisiert sind. In letzter Zeit wurde auch der Rezeptor von Relaxin entdeckt, bei dem es sich um einen G-protein coupled receptor handelt.

Vieles was heute über insulin-like Moleküle und ihre Funktionen bekannt ist, wurde durch Studien im Säuger Modellsystem herausgefunden. Durch solche Studien wurden wertvolle Erkentnisse für das Verständnis und die Behandlung von Krankheiten wie Krebs und Diabetes gewonnen.Homologe in Komponenten des Insulin Signalwegs wurden auch in niederen Organismen wie C. elegans und D. melanogaster entdeckt und beschrieben. Tatsächlich hat Drosophila bedeutend dazu beigetragen um neue Komponenten um Insulin Signalweg zu identifizieren und deren Funktion zu definieren. Allerdings sind die Insulin signal Komponenten in niederen Tieren erst seit noch nicht all zu langer Zeit bekannt und auch weniger gut charakterisiert als bei Säugetieren. Nichtsdestotrotz bietet sich die Möglichkeit durch die Erforschung des Insulin Signalwegs in niederen Tieren wichtige neue Erkenntnisse zu bisher bekanntem hinzufügen. Im Jahr 2000 sind, sieben Insulin-ähnliche Gene im Drosophila Genome identifiziert worden. Jedes Gen kodiert für ein potentielles Drosophila Insulin-like peptide, DILP 1-7. Die Expressionsmuster der *dilp* Gene zeigen, dass mindestens

drei von sieben, in besonderen Neuronen exprimiert werden. Entfernung dieser Neuronen während des Wachstums, führt zu kleinen Fliegen mit erhöhtem Zucker- und LipidSpiegel. Diese Resultate legen nahe, dass die DILPs in Drosophila eine ähliche Rolle spielen, wie insulin und die IGFs in Säugtieren. Jedoch ist die funktionelle und biochemische Charakterisierung des Insulin Signalwegs noch längst nicht abgeschlossen.

Um die unterschiedlichen Funktionen der einzelnen DILPs in der Wachstumsregulation zu studieren, wurden Sie mit Hilfe des UAS-GAL4 Systems ubiquitär exprimiert. Schwache ubiquitäre Expression hat eine Erhöhung des Körpergewichts von männlichen und weiblichen fliegen zur folge, wobei DILP 2 den stärkesten wachstumsfördernden Effekt gezeigt hat. Um die Bindungs- und Signalkapazitäten der unterschiedlichen DILPs zu erfassen und untereinander zu vergleichen, war es unerlässlich, die DILPs aufzureinigen. Vier der DILPs, 2,3,4 und 5 wurden synthetisiert und aufgereinigt anhand von standard Methoden, die für die Reinigung von menschlichem Insulin benutzt werden. Die Aktivität der DILPs wurde getestet und verglichen durch den Vergleich der phosphorylierung zweier ‚downstream' Kinasen, Protein kinase B und S6 Kinase. DILP 2 konnte jene phosphorylierung am stärkesten stimulieren, gefolgt von DILP 3, DILP 4 und DILP 5. Ebenso war die Bindungsaffinität an den Rezeptor von DILP 2 höher als von DILP 3, wie durch einen ‚Competitive binding assay' gezeigt werden konnte. Diese Resultate könnten den Unterschied in der Signalkapazität zwischen den einzelnen DILPs erklären. Flow cytometry und BrdU Einschluss Studien wurden durchgeführt, um die Effekt der DILPs auf Zellgrösse und Zellzyklus zu studieren. Die Resultate zeigen, dass DILP stimulierte Zellen, im vergleich zu unstimulierten Zellen, etwas grösser sind und sich ein höherer Prozentanteil in der S-phase befindet. Um die Rolle der DILPs in der Zuckeraufnahme zu analysieren, wurden sogennante ‚glucose uptake assays' in Zellen und Gewebe durchgeführt. Unter den getesten Konditionen, haben die DILPs keinen erkennenbaren Einfluss auf die Glucosufnahme gezeigt, was allerdings die Möglichkeit nicht ausschliesst, dass die DILPs eine solche physiologische Funktion übernehmen können. Der einzige Drosophila Insulin Rezeptor ist in verschiedenen Geweben unterschiedlich stark vorhanden, was mit der Höhe der Aktivität von Downstream Kinasen korreliert werden kann.

Zusammengefasst hat diese Studie daher dazu beitragen, das Verständnis der Funktion der einzelnen DILPs in Drosophila, in beleg aur die Wachstumskontrolle und den Stoffwechsel zu verbessern.

Chapter 1

Introduction

Insulin has undoubtedly emerged as the 'champion' molecule of modern medical research. The molecule is probably the most well studied bio-molecule in the history of scientific research and has brought many awards for pioneering work of scientists. The discovery of Insulin as a miracle drug for Diabetes, by Frederick Banting and Charles Best in 1920, was one of the landmarks in modern medicine. Frederick Banting and JJJ Macleod received the Nobel Prize for Physiology and Medicine in 1923. The production of animal insulin was started soon by Eli Lilly and Insulin was available in the market as a treatment for diabetic patients. Insulin was the first molecule whose primary structure, the sequence of amino acids, was determined by British molecular biologist Frederick Sanger. For this pioneering work, he was awarded the Nobel Prize in Chemistry in 1958. In 1967, after decades of work, Dorothy Crowfoot Hodgkin determined the spatial conformation of the molecule, by means of X-ray diffraction studies. She was also awarded the Nobel Prize in Chemistry. In 1978, the first recombinant molecule-Insulin was produced in *Escherichia coli* by Genentech.

The discovery of Insulin opened several new fields of research in biology, chemistry and medicine. Since its discovery more than 80 years ago, not only the molecular properties but also its action and functions have been studied extensively. This research has shed light on many of the important life processes which when gone wrong can be the cause of threatening diseases of the modern world- Cancer and Diabetes. More recently, a variety of insulin-like or insulin-related molecules have been discovered; not only in mammals but also in various species, and thus the number of members of the Insulin family has increased.

The Insulin Family

The Insulin/IGF/Relaxin superfamily is an ancient family of functionally diverse proteins. Members of the insulin family have been found from organisms of the lowest Phylum Porifera through Chordates, Molluscs and Insects to Mammals and humans [1].

Despite the broad magnitude of functional divergence present within the family, all proteins of the insulin family exhibit a high degree of structural conservation. The primary peptide sequence of each member of the family is characterized by three domains comprised of an amino terminal B peptide (or chain) joined to a carboxyl terminal A peptide by an intervening

C peptide (B-C-A) [2]. Between the different hormones within the family (paralogs) and similar hormones in different species (orthologs), the B and A chain peptides are relatively invariant and exhibit a pattern of distinct and highly conserved Cysteine motifs. These cysteine motifs characterize the family; specifically the motif (CC-3X-C-8X-C) present in the A peptide has been termed the Insulin signature. Many members of the insulin family of hormones are synthesized as preprohormones, with the primary peptide undergoing post-translational modification to generate a cysteine-linked heterodimer of the B and A peptides that functions as the active hormone [2].

A decade ago the Insulin family in mammals comprised four members: Insulin, IGF-I, IGF-II, and relaxin. In the recent years, additional members of the family, termed INSL3 [3, 4], INSL4 [5, 6], INSL5 [7, 8] INSL6 [9- 11], and INSL7 [12], have been identified.

Insulin

Mammalian Insulin is a 51 amino acid anabolic peptide-hormone that is translated and secreted from specialized endocrine cells, the β-cells in the Islets of Langerhans. Insulin is transcribed from a single *Ins* gene located at 11p15.5. There are two Insulin gene isoforms in rodents; *Ins1* represents a functional retroposon [13]. In adult mice, Insulin is synthesized from transcripts of both genes, but *Ins2* mRNA appears to be translated more efficiently than *Ins1* mRNA [14]. Mammalian Insulin possesses typical hallmark structure of the Insulin family.

INSL 3

Having been initially identified in boar testes [15], the product of the INSL3 gene, relaxin-like factor, or Ley-I-L (Leydig insulin-like peptide precursor), was subsequently identified in both human and mouse as a single copy gene containing two exons and a single intron [3, 16]. Human INSL3 is located on the short arm of chromosome 19 and is structurally similar to Relaxin. The major site of expression of INSL3 is in Leydig cells of the testes, but it is also expressed in the theca cells of the corpus luteum, the trophoblast, breast, and a variety of other tissues [17-20]. The first indication of an essential role for INSL3 in reproduction came from studies of knockout models in mice. *Insl3* null male mice were cryptorchid suggesting that *Insl3* gene plays a major role in the development of the gubernaculum and subsequent testicular development [4, 21]. The phenotype of the *Insl3* null mice was similar to that of the LGR8 (GREAT) null mice, leading to the identification of LGR8 as the cognate receptor for INSL3 [15, 22]. Subsequent screening of boys with cryptorchidism, the most frequent

congenital abnormality in humans, resulted in the identification of mutations within the INSL3 gene and its promoter sequence and the LGR8 gene [23-26], although the mutations in these two genes are responsible for only a small proportion (<10%) of familial cryptorchidism [27]. The contribution of abnormalities in LGR expression, cognate signaling pathways, and target genes in the pathogenesis of cryptorchidism remains to be investigated. In the testis, in addition to its actions on the gubernaculum, INSL3 suppresses germ cell apoptosis and acts in a paracrine manner as a survival factor for male germ cells [28]. Whereas much of the interest regarding the biological role of INSL3 has been focused on the testes, recent experimental observations provide clues to actions of INSL3 in the ovary. LH transiently increases INSL3 expression in theca cells of the ovary and INSL3 suppresses intra-oocyte cAMP levels with stimulation of induction of oocyte maturation, suggesting a paracrine role for INSL3 in mediating preovulatory LH actions on the ovary [28].

INSL 4

Efforts directed at identifying differentially expressed genes within the human placenta led to the identification of the product of the INSL4 gene, early placenta insulin-like peptide (EPIL) or placentin [5, 6]. In contrast to other members of the insulin super family, INSL4 is primate-specific as INSL4 nucleic acid sequences are absent in rodent, horse, and lemur genomes [29]. Structurally, the gene is similar to Insulin, Relaxin, and INSL3 and contains two exons and a single intron. It is located on 9p24, where it is clustered with the two human relaxin genes [30]. The predominant site of expression of the INSL4 gene is in the placenta during early pregnancy, but it is also expressed to a lesser degree in interbone ligaments, perichondrium, and the uterus [31]. Given that it is detectable in maternal serum during pregnancy, INSL4 presumably functions as a hormone. It is also likely that INSL4 has paracrine and autocrine actions. There is a paucity of information regarding the cognate receptor(s) for INSL4, although conditioned medium from cells overexpressing INSL4 increases total cellular tyrosine phosphorylation via a pathway distinct from that of the insulin receptor [6]. Recent reports of INSL4 being overexpressed and secreted in *erb-3*-positive breast cancer cells with high invasive potential and in hydatiform moles suggest a role for INSL4 in tissue invasiveness and cell migration [32].

INSL 5 AND INSL 6

Three laboratories, working independently identified, two new genes: INSL5 (relaxin/insulin-like factor 2, RIF2) and INSL6 (relaxin/insulin-like factor 1, RIF1) [7-10]. Human, mouse, and rat orthologs of these genes have also been identified. Both genes encode proteins that are

clearly members of the Insulin super family; they contain signal peptides and exhibit the requisite cysteine motifs. The human INSL5 gene is located on chromosome 1 and the orthologous mouse *Insl5* is located on mouse chromosome 4. Human *Insl6* is located in the region 9p24 in proximity to the human relaxin genes as well as to *Insl4*. The mouse ortholog, *Insl6*, is located on chromosome 19, the chromosome that also contains the single mouse relaxin gene. In humans, INSL5 is maximally expressed in the uterus and the digestive tract, with highest levels of expression in the rectum. In the mouse, expression of mouse *Insl5* is described in thymus, kidney, heart, brain, and testis. INSL6 is maximally expressed in germ cells of the testis, with lower levels of expression detectable in a variety of other tissues including intestine, thymus, kidney, uterus, ovary, spleen, breast, lung, and liver [10, 33]. The INSL6 peptide undergoes posttranslational modifications including glycosylation and ubiquitination [33]. A paracrine role for INSL6 in modulating Sertoli cell function is suggested by studies that demonstrate augmentation of FSH-stimulated cAMP in Sertoli cells [34].

The discovery of these new ligands spurred efforts to identify their cognate receptors. These efforts resulted in the discovery that LGR8, a member of the LGR (leucine-rich repeat-containing *G* protein-coupled *r*eceptors) subfamily of GPCR (*G p*rotein-*c*oupled *r*eceptors) receptors, was the cognate receptor for INSL3 [35]. This will be further discussed in the following sections.

Relaxin

The peptide hormone relaxin (RLX) was one of the first hormones to be described, in the 1920s, by Frederick Hisaw [36]. Its crystal structure is similar to that of Insulin, whereby the α helices of the A and B chains mutually support the conformation of each other, and are held together by two inter chain cysteine bridges and one intra chain bridge in the A chain. Similar to Insulin, mature RLX appears to be the result of post-translational processing, with convertase-like enzymes releasing the heterodimer and a connecting or C-peptide from a single longer precursor protein. Very little is known about these processing events and, in pregnancy, an unprocessed pro-form also appears to be secreted into the blood [37], although the physiological relevance of this is unclear. In the past ten years, there has been a reappraisal of RLX physiology. It is now apparent that the function of RLX to prepare the birth canal for parturition (cervical softening, widening of the pubic symphysis and inhibition of spontaneous myometrial contractions) is a special endocrine function that is found in some species, such as rodents and carnivores, but not in others (e.g. cows, sheep and humans). In ruminants, it appears that the *rlx* gene (officially *RLN1*) responsible is partially deleted [38,

39], but in primates the reduced importance of RLX in peripartum physiology has focused the attention on other possible roles for RLX, which had previously gone unnoticed, and which now appear to be very significant. RLX might support embryo implantation [40], and it also acts as a cardiovascular hormone. Relaxin has also been shown to be responsible for permitting the increased blood volume in pregnancy, and acting in the brain to reset the vasopressin response to hyperosmolarity [41]. Although several studies have been published on the growth effects of relaxin, most show only a small effect on growth parameters, with inhibitory effects at high relaxin concentrations and sometimes stimulatory effects at low concentrations [42].

Insulin- like growth factors (IGFs)

Early studies showed that growth hormone did not directly stimulate the incorporation of sulfate into cartilage, but rather acted through a serum factor, termed 'sulfation factor,' which later became known as 'somatomedin' [47]. Three main somatomedins have been characterized: somatomedin C (IGF 1), somatomedin A (IGF 2), and somatomedin B [48, 49].

Human IGF 1 is a single chain 70-amino acid polypeptide cross-linked by three disulfide bridges, with a calculated molecular mass of 7.6 kD [50]. The IGF 1 protein displays homology to proinsulin. IGF 1 is synthesized as a precursor protein that undergoes proteolytic processing at both ends [51]. *Igf1* and *Igf2* genes have complex structures with multiple promoters [53]. The expression of both genes is regulated at the levels of transcription, RNA processing, and translation.

Insulin-like growth factor 2 (IGF 2) is a 7.5 kDa, 67 amino acid peptide which is thought to mediate some of the actions of growth hormone. IGF 2 peptide consists of the A, C, and B chains, and is structurally homologous to IGF 1 and pro Insulin.

Both IGF 1 and IGF 2 are composed of four domains (B, C, A and D). The B and A domains of each peptide show a high degree of sequence identity with each other and with the B and A domains of proinsulin. The C domains are structurally dissimilar, and the D domains are unique to IGF 1 and IGF 2.

Receptor-ligand functions

There are at least three separate receptors that interact with these hosts of ligands: Insulin receptor [53, 54], IGF 1 receptor [56], and IGF 2 receptor [57]. A fourth member of the family, Insulin receptor-related receptor (IRR) [58], is as yet orphaned, although its ability to bind all the various Insulin-like peptides has not been extensively tested.

Three of the four receptors (IR, IGF1R, and IRR) belong to the family of ligand-activated receptor tyrosine kinases. Unlike other receptor tyrosine kinases, these receptors exist at the cell surface as homodimers composed of two identical α/β-monomers, or as heterodimers composed of two different receptor monomers (*e.g.*, $IR_{\alpha\beta}$/ $IGF1R_{\alpha\beta}$, or $IR_{\alpha\beta}$/ $IRR_{\alpha\beta}$). Upon ligand binding, they undergo a conformational change, which enables them to bind ATP and become autophosphorylated [60, 61]. Autophosphorylation increases the kinase activity of Insulin receptor-type receptors by three orders of magnitude, enabling them to phosphorylate a number of substrate proteins and engender growth or metabolic responses [61]. It is likely that this receptor family contains additional members: there is evidence for a separate IGF 2 receptor regulating placental growth [62-66], and for an insulin-like peptide receptor [63]. Unlike IR, IGF1R, and IRR, the product of *Igf2r* is not a tyrosine kinase. Instead, it is a monomeric receptor with a large extracellular domain made up of 15 repeat sequences and a small region homologous to the collagen-binding domain of fibronectin. IGF2R functions also as the cation independent mannose-6-phosphate receptor [57]. IGF2R does not have a signaling domain and is thought to be recycled between the plasma membrane and intracellular compartments. Based on the *in vivo* mutagenesis experiments described below, it is now clear that IGF 2 binding to IGF 2R serves as a mechanism to clear circulating IGF 2, rather than as a signaling mechanism.

IGFs and IGFRs in humans

The *Igf1* locus has been extensively analyzed in several groups of children with "idiopathic" congenital growth retardation; however, no mutations have been identified, leading to the suggestion that IGF 1 mutations are not a common cause of growth retardation in humans [72–75]. The debate has been rekindled by the identification of a single case of human *Igf1* knockout due to a partial deletion of *Igf1*. This patient strikingly resembles the phenotype of *Igf1*-deficient mice, with severe prenatal and postnatal growth failure [76]. The patient showed poor growth during gestation, infancy and childhood. His growth was retarded and the patient presented with sensor neural deafness and mental retardation.

IGF and IGFRs in mice

Nullizygous animals for *Igf1* and *Igf1r* are born with Mendelian frequency, suggesting that *Igf1* and *Igf1r* are not required for successful completion of gestation. The birth weight of *Igf1* null mice is 60% of normal; that of *Igf1r* nulls is 45% [64, 65, 67]. Survival of *Igf1* null mice is strain dependent and is associated with postnatal growth retardation. Prenatally, IGF-I mediates growth independently of GH; postnatally, GH is required for hepatic IGF-I synthesis and mediates approximately 50% of IGF-I action on growth [68].

In contrast to *Igf1* mutants, *Igf1r*-deficient mice invariably die within minutes of birth, probably as a result of respiratory failure. Mice are born with multiple abnormalities, including muscular hypoplasia, delayed ossification, and thin epidermis [65]. *Igf1r* null mice have also been reported to develop metabolic abnormalities. These include mild hyperglycemia and decreased β-cell mass [69], although the latter was reportedly normal in other studies [70]. Since IGF 1R shares many signaling properties with IR [71], these findings are not altogether surprising.

Igf2 mutants are approximately 60% of normal size at birth. However, their postnatal growth is unaffected, consistent with a role of *Igf2* in embryonic growth and with the lack of *Igf2* expression in adult mice [77, 78, 79].

The phenotype of *Igf2* mutant mice is in stark contrast with that of *Igf2r* mutants. When mice inherit the *Igf2r* null allele through the maternal route, they show increased serum and tissue levels of IGF 2, associated with an approximately 40% increase in size by weight and generalized organomegaly with heart abnormalities, kinky tails, postaxial polydactyly, and edema [80, 81]. A similar phenotype is observed in true homozygous knockouts [82]. *Igf2r*-deficient mice usually die perinatally and rarely survive to adulthood. The elevation of IGF 2 levels in these mice suggests that *Igf2r* is important for IGF 2 clearance, and that failure to remove IGF 2 from the circulation results in developmental abnormalities [82, 80, 81].

The lethal phenotype due to IGF 2 induced overgrowth in *Igf2r* mutant mice can be rescued by a homozygous null mutation of *Igf1r* [82]. This experiment indicates that IGF 2 signaling through IGF1R is responsible for the developmental abnormalities found in *Igf2r*. In contrast, in *Igf1r/Igf2r* mutant mice there are no developmental abnormalities. This finding indicates that IGF 2 signaling through IR is sufficient to engender growth, but insufficient to induce lethal embryonic abnormalities [82].

Insulin Receptor (IR)

The existence of a specific receptor for insulin was first proposed by Roth and co-workers [85]. Cloning of the receptor cDNA [86, 87] and gene [88] ushered in molecular investigations of insulin action, with the identification of insulin receptor mutations in humans with extreme insulin resistance [89], the determination of the crystal structure of the receptor kinase [60, 90], and the development of pharmacological agents that enhance receptor signaling to treat diabetes [91]. The generation of mice bearing insulin receptor mutations has been instrumental in dissecting the pathogenesis of insulin resistance, diabetes, and obesity [93–101].

Insulin Receptor in mice

Mice lacking *Ir* are born at term with slight growth retardation (~10%) [63]. With the exception of a marked hypotrophy of the adipose tissue [102], their embryonic development is unimpaired. After birth, metabolic control rapidly deteriorates: glucose levels increase upon feeding, despite Insulin levels approximately 100- to 1,000-fold higher than in normal littermates. β-cell failure occurs within a few days, characterized by the disappearance of insulin storage granules within the β-cell cytoplasm and followed by death of the animals in diabetic ketoacidosis. This experiment indicates that *Ir* is necessary for postnatal, but not for prenatal, fuel homeostasis.

Insulin Receptor in Humans

Mutations of *Ir* in humans are phenotypically heterogeneous: the severity of the syndrome runs the gamut from mild insulin resistance [103-104] to leprechaunism [105-107]. The latter represents the severest form of insulin resistance due to *Ir* mutations, and, in four separate cases, has been shown to be caused by functional *IR* knockouts [108-111]. As in mice, lack of the IR in humans is compatible with embryonic development and term birth. However, the similarities between the two species are limited [112, 113]. Humans lacking IR are severely growth retarded at birth and gain little if any weight thereafter [105-111, 114].

The likeliest explanation of the difference between *Ir* deficient mice and children with leprechaunism is that the embryonic growth of humans and rodents follows different patterns. Rodents are born comparatively earlier than humans, at a stage corresponding to 26 weeks of human gestation. Not only are rodents born developmentally “earlier” than humans, their body composition at birth is quite different [115].

Insulin receptor-like Receptor (IRR)

IRR is the only known orphan receptor of the Insulin receptor family [116]. Despite extensive investigations, its ligand remains unknown [117-120]. It is unclear whether IRR functions as an independent homodimeric receptor or whether it functions primarily by engaging in heterodimer formation with IR and IGF1R [119, 121], similarly to ErbB-2 in the epidermal growth factor receptor family [122, 123]. *Irr* transcripts are predominantly found in kidney, neural tissues, stomach, and pancreatic β-cells [122, 124–129]. Mice lacking IRR are phenotypically normal; double knockouts of *Irr* and *Ir* are phenotypically identical to *Ir* knockouts [130]. Thus, the function of IRR remains unclear. Nef et al. in 2003, showed IR, IGF-1R and IRR, are required for the appearance of male gonads and thus for male sexual differentiation. Genotypic male mice that are mutant for all three receptors develop ovaries and show a completely female phenotype [131].

Insulin and Insulin Receptor in *Caenorhabditis elegans*

The C. elegans insulin receptor (DAF-2) was known due to its dauer arrest and increased longevity phenotypes [132]. In 1997, Ruvkun and colleagues showed that *daf-2* encodes an Insulin receptor family homolog [133]. Until recently little was known about the nature of the DAF-2 ligand(s). Using a combination of sequence and structure based algorithms to screen the *C. elegans* genome, 37 candidate genes encoding insulin-like peptides were identified [134]. The insulin-like peptides fall into four basic classes. Despite extensive sequence variation, computer modeling of peptide tertiary structure suggests that each class can adopt the canonical insulin-fold motif characterized by two parallel α-helices in the A chain crossed by a single β-helix in the B chain [135]. While mutations affecting many downstream components of the insulin signaling pathway in *C. elegans* have been identified and characterized, none affecting the insulin ligands were known. Since 37 members of the insulin- like gene family are identified in the *C. elegans* genome, the potential for functional redundancy suggests that such mutations may be difficult to identify. However, among the 37 insulin genes, *daf-28* is so far the only insulin mutant to affect dauer arrest. *daf-28* was revealed from this functional redundancy by a dominant-negative allele that disrupts a probable proteolytic processing site required for insulin maturation [136]. Some Insulin-like ligands are proposed to function as agonists or antagonists of DAF-2, the *C. elegans* insulin receptor [134].

Insulin and Insulin Receptor in *Drosophila melanogaster*

Loss of function mutations in the *dinr* gene render pleiotropic recessive phenotypes that lead to embryonic lethality. The Drosophila Insulin receptor (dInR) was identified by Fernandez et. al. [137], Chen et. al. [138] and Ruan et. al. [139] in 1995-96. They showed that the dInR proreceptor (Mwt- 280 kDa) is processed proteolytically to generate an Insulin-binding alpha subunit (Mwt- 120 kDa) and a beta subunit (Mwt- 170 kDa) with a protein tyrosine kinase domain. The dInR β (Mwt- 170 kDa) subunit contains a novel domain at the carboxyterminal side of the tyrosine kinase, in the form of a 60 kDa extension which contains multiple potential tyrosine autophosphorylation sites. This 60 kDa C-terminal domain undergoes cell-specific proteolytic cleavage which leads to the generation of a total of four polypeptides (α-120, β-170, β-90 and a free 60 kDa C-terminus) from the *dinr* gene. Using the conserved spacing of four cysteines within the A chain as a signature for insulin-like peptides, Brogiolo et al. identified seven predicted genes matching the above criteria, termed *dilp1–7* for Drosophila insulin-like peptides 1-7 [140]. The topic will be dealt in more detail in the following Chapters.

LGR

LGRs are mosaic proteins that contain an extracellular domain with multiple leucine-rich repeats (LRRs), important in ligand binding, and a GPCR transmembrane domain. Studies of LGRs from different species suggest that three LGR subtypes (A, B and C) evolved during the early evolution of metazoans and that each subtype of LGR shares a similar LRR domain and a unique hinge region between the LRR and the transmembrane region. The Type A LGRs include the follicle-stimulating hormone receptor (FSHR), the luteinizing hormone receptor (LHR) and the thyroid-stimulating hormone receptor (TSHR), important for signaling of the heterodimeric glycoprotein hormones FSH, LH, and TSH, respectively. In mammals, the Type B LGR comprises three members, LGR4–6, which remain orphan GPCRs at the present time. By contrast, Type C LGRs have only two members, LGR7 and LGR8.

Based on the comparison of phenotypes of mice deficient in *Insl3* or *Lgr8*, it was hypothesized that the relaxin family peptides could function as cognate ligands for Type C LGRs [141]. Indeed, functional characterization has established that relaxin activates both LGR7 and LGR8 [141]. The finding that two orphan GPCRs are the receptors for relaxin is surprising. As a member of the insulin superfamily of peptide hormones, one would have thought that relaxin would activate a receptor similar to those for insulin and the IGFs.

Instead, in spite of their structural similarity, relaxin and insulin family peptides belong to two independent signaling pathways: one is coupled with the GPCRs and the other activates single-transmembrane tyrosine kinase receptors. Following the identification of LGR7 and LGR8 as relaxin receptors, the closely related Relaxin 3 and INSL3 have been shown to function as selective agonists for LGR7 and LGR8, respectively [44, 45]. In addition, the ectodomains of these LGRs are important for ligand binding, similar to that found for Type A LGRs [142-144]. These data have brought about a better understanding of relaxin physiology and provide a basis for the future molecular and pharmacological characterization of this unique signaling system.

Insulin-like growth factor binding proteins (IGFBPs)

The insulin-like growth factor-binding proteins (IGFBPs) represent a family of conserved proteins that share the ability to bind the insulin-like growth factors, IGF 1 and IGF 2, with affinities comparable to their respective receptors. Unlike the transmembrane IGF receptors, the IGFBPs are secreted proteins. Six high-affinity members of this family of proteins (IGFBP 1-6) have been identified and the cognate cDNAs for these binding proteins have been cloned from a variety of species [145]. IGFBPs are important molecules for regulating the bioavailability of IGF 1 and IGF 2 to the receptors. The synthesis and abundance of IGFBP are regulated by growth hormone or serine proteases which can cleave IGFBPs [146]. The IGFBPs also regulate IGFs bioavailability by maintaining a circulating reservoir of IGFs and prolonging their half-life. More than 99% of the circulating IGFs are bound to IGFBPs, and at least 75% of the bound IGF is carried as a 150 kDa trimeric complex composed of IGF, IGFBP and Acid-labile subunit [147, 148].

Insulin-IGF signaling pathway

IGF 1 binding to the IGF 1 receptor activates the receptor's intrinsic tyrosine kinase activity, resulting in autophosphorylation and tyrosine phosphorylation of members of the Insulin Receptor Substrate (IRS) family [149, 150]. Migration of IRS to the plasma membrane is mediated via the pleckstrin-homology (PH) domain, and docking with the phosphotyrosines on the IGF 1 receptor β-subunit tyrosine kinase via the IRS phosphotyrosine-binding (PTB) domain [151]. Members of the IRS family (IRS 1-4) in mice, all contain conserved N-terminal PH and PTB domains, but they differ in variety and number of tyrosine phosphorylation Src-homology domain-2 (SH2-domain) docking sites in the C-terminal tails. Tyrosine-phosphorylated IRS is then able to recruit the 85 kDa regulatory subunit of

Phosphatidylinositol-3-kinase (PI3-K) via its SH2 domain, leading to activation of the enzyme [149-151] to convert phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 then recruites protein kinase-B (PKB, also known as Akt), 3-Phosphoinositide-dependent protein kinase-1 (PDK1) [137] and isoforms of protein kinase-C (PKC) [153, 154]. PDK1 then activates PKB and certain atypical PKC isoforms by serine phosphorylation [154, 152]. Activated PKB then inactivates glycogen synthase kinase-3 (GSK3) via serine/ threonine phosphorylation and, in contrast, activates another protein kinase, the mammalian target of rapamycin (mTOR), leading to downstream phosphorylation and activation of the 70 kDa-S6 kinase (p70-S6K) [155, 154]. PKB can also directly serine/threonine phosphorylate Forkhead transcription factors which, in turn, leads to regulation of several genes [156, 157]. IGF 1 mediated tyrosine phosphorylation of IRS also engages the adaptor molecule growth factor receptor-bound protein-2 (Grb2) via its SH2 domain to a phosphotyrosine site on IRS [149-150]. This, in turn, leads to an increased binding of IRS-docked Grb2 to the murine son-of-sevenless-1 protein (mSOS), a guanine nucleotide exchange factor which converts inactive Ras-GDP into active Ras-GTP [149, 150]. Activated GTP-bound Ras then recruits the Raf serine kinase which, in turn, phosphorylates mitogen-activated protein (MAP)-kinase-kinase (MEK), resulting in MEK-mediated phosphorylation and activation of the MAP kinase (MAPK; erk 1 and 2 isoforms) [150]. Activated MAPK can then activate other protein kinases such as the p90 ribosome S6 kinase (p90RSK), or migrate to the nucleus where it may phosphorylate certain transcription factors, leading to mitogenesis [158]. IGF can also activate the Ras/MAPK branch of the pathway independently of IRS, via IGF 1 receptor kinase tyrosine phosphorylation of the SH2-containing protein (Shc) which then directly binds Grb2 to mSOS, resulting in activation of Ras/MAPK [149, 150]. Whichever signal transduction pathway is mediated, activation of MAPK and PI3-K are known to be a requirement for induction of mitogenesis in most mammalian cell types [149, 150].

The unique contribution from the Drosophila system came from the application of genetic screens for modifiers of cell growth, which had already identified several proximal components of the IR/IGF-1R pathway (e.g. Chico (IRS-1), PI-3K, PKB, PTEN) as important regulators; the more recent studies have revealed the elements intervening between PKB and TOR. In 2001, several groups identified the tumor suppressor protein Tuberin or TSC2 as a negative regulator of cell growth [159-160]. Mutations in the human Tuberin gene are responsible for a major portion of the disease Tuberous Sclerosis, with the remainder of instances attributable to mutations in a protein Hamartin, or TSC1; the two polypeptides function as an obligatory heterodimer [162, 163]. Although earlier studies in Drosophila screens had identified TSC1 as a negative regulator of cell overgrowth, the connection to the

IR/IGF-1R pathway was not initially appreciated. Biochemical and genetic studies in Drosophila [160, 161, 164, 165] rapidly established that the TSC complex was positioned in the IR/IGF-1R pathway downstream of PI3K and PKB, which suppress its inhibitory effects on growth, but upstream of the p70 S6 kinase. This was followed rapidly by studies in mammalian cells, both in culture and from TSC deficient mice, which showed that TSC1/2 overexpression inhibits S6K and this inhibition is ameliorated by active PI-3 K through PKB. Reciprocally, TSC1 or 2 deficiency is accompanied by constitutive upregulation of S6K, which remains sensitive to inhibition by rapamycin but exhibits resistance to inhibition by withdrawal of ambient amino acids [163-165]. These results indicated that the TSC complex functions as an inhibitor of TOR, and relief of TOR from this inhibition makes cells resistant to the negative regulation of S6K by amino acid withdrawal. Interestingly, Caenorhabditis elegans exhibits a canonical IR/IGF-1R pathway, namely a single receptor, DAF-2 that controls a Type 1 PI-3 kinase, PDK1 and PKB, but this pathway does not appear to have developed strong connections with the TOR pathway [166]. Notably, the C. elegans genome lacks homologs of TSC1 and TSC2.

Within months of the discoveries concerning TSC2, Drosophila screens provided the identity of the intermediary between the TSC complex and TOR by the discovery of the small GTPase Rheb as a positive regulator of cell growth [167-168]. Rheb deficiency inhibits the stimulation of cell growth caused by overexpression of PKB or by the absence of TSC; conversely, the ability of Rheb to promote cell growth requires S6K but is unaffected by mutations that inactivate PKB and upstream components of the InsR/PI-3K pathway. The carboxy terminus of TSC2 contains a domain that resembles the GTPase activator domains for Ras-like GTPases, and biochemical studies demonstrated that TSC1/2 strongly promotes the conversion of Rheb-GTP into Rheb-GDP [170-174], providing a satisfying mechanistic explanation for the inhibitory action of the TSC complex on cell growth, that is deactivation of Rheb. Thus, a substantial body of genetic and biochemical evidence has established that the InR/PI3K pathway bifurcates downstream of PKB, with one limb proceeding through the regulation of the TSC complex and the activation state of the Rheb GTPase to control the TOR pathway and thus affects translational efficiency of many genes that control cell growth.

In contrast to the substantial advances in understanding the mechanisms by which the cellular energy charge and the InR/PI-3K pathway control TOR signaling, much less information is available as to the molecular elements that enable regulation of TOR signaling by amino acids. Perhaps the most significant advance has emerged from the identification of a set of polypeptides that are physically associated with the TOR kinase and are necessary for TOR signaling. Work in both the yeast [175] and mammalian [178-182] systems identified the

existence of two TOR complexes of distinct polypeptide composition. One complex, TORC1, contains TOR, LST8/GbL and KOG1/raptor, whereas the second complex, TORC2 contains TOR, LST8, AVO3/rictor (and, in yeast, AVO1 and AVO2). The TORC2 complex regulates the actin cytoskeleton through a PKC in a rapamycin-insensitive manner [175, 181, 182]; mammalian TORC2 does not regulate S6K or 4E-BP. LST8 binds to the TOR catalytic domain and RNAi-induced reduction of LST8 reduces S6K activity *in vivo*. Overexpression of LST8 does not alter TOR signaling *in vivo* or rescue S6K from inhibition by amino acid withdrawal (unlike Rheb), but does cause a 4–8-fold increase in the TOR kinase activity assayed *in vitro* [180]. As with LST8, raptor is absolutely required for TOR signaling to S6K and 4EBP and mRNA translation; RNAi-induced elimination of raptor in C. elegans reproduces essentially all features of the TOR-deficiency phenotype [174]. The importance of raptor to TOR kinase activity is attributable to raptor's ability to bind S6K and 4E-BP1; thus, raptor does not alter TOR's catalytic activity, but serves as the substrate-binding subunit of the TORC1 complex, whose presence promotes strongly (in the case of S6K) or is absolutely required (in the case of 4E-BP) for mTOR-catalyzed phosphorylation of these substrates, *in vitro* and probably *in vivo* as well [178, 179].

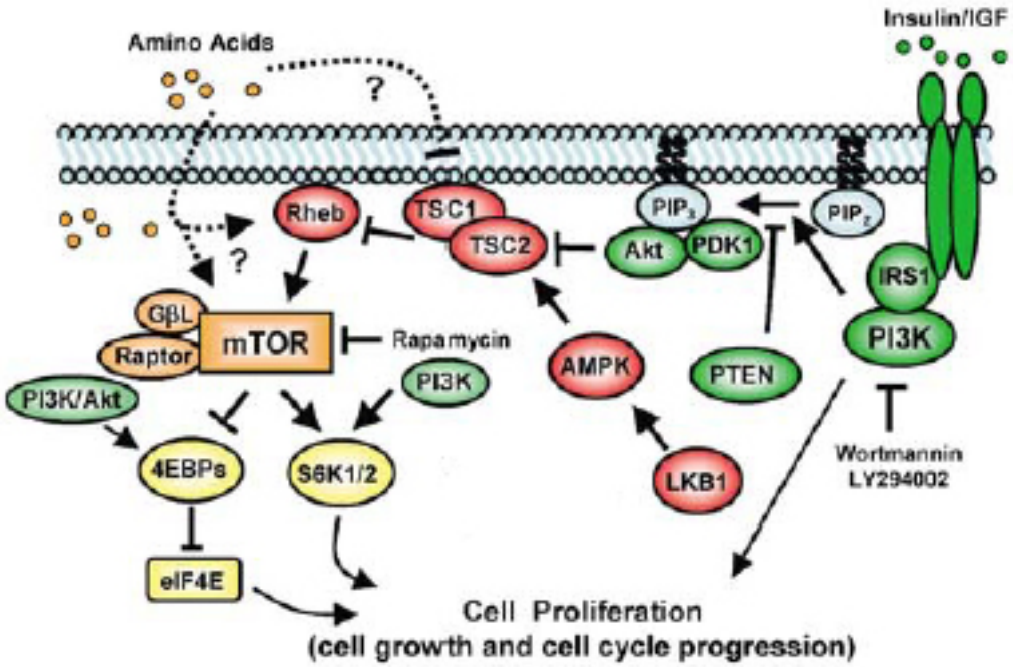

Figure 1 (adapted from Ref [180]) shows schematic representation of the Insulin/IGF signaling pathway.

Insulin/IGF signaling and metabolism

The amount of information available on metabolism mechanisms, and the role of Insulin and other hormones in regulating it, is huge. Still the regulation and control of metabolism is not fully understood. Almost all of the knowledge on understanding energy metabolism comes from studies in mammalian systems. It is beyond the scope of this thesis to handle the subject in detail. Hence a short overview of energy metabolism in mammalian systems is given, paying main attention to the role of Insulin in it.

Our bodies are an integrated system of organs, each with its own requirements for nourishment and energy utilization. In spite of this, our tissues share a common circulation system. Strict limits on the blood levels of ions, lipids and sugars must be upheld if a healthy situation is to be maintained.

Integration of metabolism is essential on both short-term and long-term basis. Perhaps the most crucial short-term element is maintenance of a stable blood glucose level. Maintenance of glucose levels over 2.5-3 mmol/s is essential for brain function. Physiological processes adjust carbohydrate and fat metabolism such that blood glucose values do not fall markedly. Integration of metabolism is important on a long-term basis too. High blood levels of glucose over a period of time lead to protein denaturation and the development of blindness, neuropathy and the kidney damage seen in Diabetes. High blood sugar levels also lead to increased circulating triglycerides and are responsible for the development of cardiovascular diseases [180].

Integration of metabolism and control by hormones and metabolites normally prevent these adverse effects of sugar. This kind of control requires hormone regulation of many processes. The main actors here are insulin, glucagon, adrenaline and growth hormone. Many other hormones control appetite and secretion of these "key" hormones. Comprehension of the mechanisms at work is difficult because these enzymatic processes and hormonal control are tightly integrated. Furthermore, different tissues have their own complicated and unique enzymatic systems.

The energy for all life processes is stored and provided in the form of high energy phosphate bond in Adenosine tri-phosphate (ATP) [183]. Stable ATP levels are maintained by a constantly shifting synthesis of ATP and precisely balancing energy use. Adenylate cyclase,

Creatinine phosphokinase aerobic and anaerobic metabolism of Glucose and fatty acids are responsible for maintaining ATP levels [184].

Effect of Insulin on glucose homeostasis

Following a meal and uptake from the small intestine or as a result of stimulation of glucose release from the liver, insulin is secreted into the blood. The increased glucose levels stimulate pancreatic secretion of Insulin. This has several immediate effects: 1. Increased skeletal muscle glucose uptake [185] 2. Inhibition of hepatic gluconeogenesis and glycogenolysis and stimulation of glucose uptake in the liver. 3. Inhibition of lipolysis in adipose tissue [186].

Skeletal muscles, which make up over 50% of the body, use Glucose as a substrate for aerobic glycolysis. During low levels of glucose in blood stream, for example before a meal, the skeletal muscles use circulating fatty acids for energy. Insulin signaling stimulates glucose uptake and its metabolism in muscles [185] and at the same time inhibits fat cells from releasing fatty acids [186]. Muscles generally take up and use fatty acids in proportion to the amount of fatty acids in blood. Free fatty acids reduce insulin's effect on glucose uptake. Free fatty acids are involved in "insulin resistance". Chronically increased serum fatty acids are implicated in the development of diabetes type 2, where a decreased response to insulin is observed, often counteracted by markedly increased insulin levels.

Control of Lipolysis by Insulin in Fat Cells

The "stress" hormone group which includes Adrenalin, Nor-adrenalin and Growth hormone activates lipolysis through a common mechanism. Glucagon also shares this mechanism. All of these, by combining with their specific receptors, activate adenyl cyclase and increase the adipocytes content of 5-cyclic AMP (cAMP). This in turn activates protein kinase A (PKA) [187]. The subsequent phosphorylation of hormone-sensitive lipase (HSL) initiates splitting of triglycerides and efflux of free fatty acids to the circulation [188, 189]. Insulin opposes this phosphorylation by down-regulating formation of cAMP and by activating a protein phosphatase (phosphodiesterase 3B, PDE 3B) which dephosphorylates hormone-sensitive lipase [190-192]. Recent studies strongly suggest that the insulin-stimulated kinase that phosphorylates PDE-3B is protein kinase B [193].

Control of Carbohydrate Metabolism by Insulin

Regulation of carbohydrate metabolism is best studied in hepatic tissue. All of the hormone and enzyme functions that control carbohydrate metabolism are found in the liver. Insulin suppresses hepatic glucose output by stimulating glycogen synthesis and inhibiting glycogenolysis and gluconeogenesis. Increased rates of hepatic glucose production result in the development of overt hyperglycemia, especially fasting hyperglycemia, in patients with type 2 diabetes [194]. Insulin exerts direct effect on the liver as well as influences the substrate availability and fluxes of free fatty acids [195, 196]. There are several important enzymatic checkpoints that act to control hepatic glycolysis and glycogen synthesis (glucokinase, glycogen synthase kinase-3), glycogenolysis (phosphorylase), gluconeogenesis (phosphoenolpyruvate carboxykinase, fructose 1,6 bisphosphatase), or steps that are common to both the pathways (glucose-6-phosphatase). Some of these check points are directly controlled by insulin signaling via phosphorylation and dephosphorylation.

Glycogen synthase kinase 3 (GSK-3) is a cytoplasmic serine/threonine kinase that plays key roles in insulin signal transduction and metabolic regulation [198-200]. This enzyme also has a key role in Wnt signalling that is critical for determination of cell fates during embryonic development [198]. In the insulin signalling pathway, GSK-3 is active in the absence of Insulin and it phosphorylates (and thereby inhibits) glycogen synthase and several other substrates. Insulin receptor activated phosphorylation cascade leads to inhibitory phosphorylation of GSK-3 by PKB/Akt. Thus, insulin activates glycogen synthase by promoting its dephosphorylation through the inhibition of GSK-3 [201].

The expression of a number of genes important for glycolysis, glycogenolysis, and gluconeogenesis is under the concerted control of insulin, glucagon, and glucocorticoids [202]. Recent studies indicate that forkhead family of transcription factors (FKHR) are phosphorylated in an insulin-dependent manner by PKB [203-211]. FKHR is a transcriptional enhancer that regulates genes involved in glucose production, cell cycle regulation, and apoptosis. Under basal conditions, FKHR resides in the nucleus. Upon insulin stimulation and phosphorylation by PKB, FKHR is excluded from the nucleus, thereby providing a powerful mechanism by which insulin could down-regulate a number of genes including IGF-binding protein-1, phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase. PGC-1 represents another transcriptional coactivator that plays an important role in the regulation of genes involved in hepatic gluconeogenesis [211, 212].

Control of Uptake and Oxidation of Fatty Acids

Metabolism of fatty acids differs from tissue to tissue. The liver can both synthesize and oxidize fatty acids. Malonyl-CoA is the main regulator of uptake and oxidation of fatty acids in mitochondria. Its formation is the opening step in the synthesis of fatty acids from carbohydrates. Acetyl-CoA carboxylase, the enzyme that catalyzes malonyl-CoA formation is strongly inhibited through phosphorylation by 5′-AMP-activated protein kinase (AMP kinase) [212]. An increased level of AMP can therefore turn on fatty acid oxidation and mitochondrial synthesis of ATP. At the same time, the increased oxidation of fatty acids tends to turn off carbohydrate oxidation.

AMPK is thought to be a major controller of many metabolic processes in addition to fat oxidation. The fuel-sensing enzyme has a major role in the regulation of cellular lipid and protein metabolism in response to stimuli such as exercise, changes in fuel availability and the adipocyte-derived hormones leptin and adiponectin [213] AMPK is a heterotrimer consisting of α-, β- and γ-subunits, each of which has at least two isoforms. It has been described in plants, yeast and mammalian cells, and was the first kinase that was shown to be regulated by changes in the cellular energy state [214]. Decreases in the cellular energy state, as reflected by an increase in the AMP:ATP ratio, cause conformational changes in AMPK that make it susceptible to phosphorylation and activation by an AMPK kinase [214]. Once activated, AMPK exerts direct effects on specific enzymes and transcriptional regulators, stimulating multiple events that enhance ATP generation and inhibiting others that consume ATP but are not necessary acutely for survival. The enzymes inhibited by AMPK include mammalian homolog of target of rapamycin (mTOR), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), and glycerol phosphate acyltransferase (GPAT), which are key regulators of protein, fatty acid and glycerolipid synthesis, respectively [214]. Tumor suppressor LKB1 has been shown to be able to phosphorylate AMPK in vitro exactly at the threonine residue which gets phosphorylated when cellular ATP levels fall. [216-218].

Hence, Insulin is essential for maintaining glucose homeostasis and regulating carbohydrate, lipid, and protein metabolism. And dysfunction in any of the signalling components of the Insulin signalling, or in the production and secretion of Insulin can cause serious disorders like Diabetes, Obesity, Hypertension and Cardiovascular problems.

Insulin and Diabetes

Diabetes mellitus is a medical disorder characterized by varying or persistent hyperglycemia (high blood sugar levels) resulting from the defective secretion or action of Insulin. There are two predominant forms of diabetes.

Type 1 diabetes (formerly known as insulin-dependent diabetes) is most commonly diagnosed in children and adolescents, but can occur in adults, as well. It is characterized by β-cell destruction, which usually leads to an absolute deficiency of insulin. Most cases of type 1 diabetes are immune-mediated characterized by autoimmune destruction of the body's β-cells in the Islets of Langerhans of the pancreas, destroying them or damaging them sufficiently to reduce insulin production. However, some forms of type 1 diabetes are characterized by loss of the body's β-cells without evidence of autoimmunity. Lifestyle does not affect the probability of getting Type 1 diabetes. In Type 2 diabetes insulin levels are initially normal or elevated, later falling, but peripheral tissues lose responsiveness to insulin (known as "insulin resistance"). Type 2 diabetes is a more complex problem than type 1 but is often easier to treat, since insulin is still produced, especially in the initial years. This is due to ‘β-cell compensation’ where the mass of beta cells increase in order to compensate for the decrease in insulin sensitivity in peripheral tissues. Type 2 diabetes is initially treated by changes in physical activity, diet and through weight loss.

Cell size, cell division and Insulin/IGF signaling

Over the last few decades, studies in different model systems have allowed great progress in deciphering the genetic networks that coordinate events such as cell-fate specification, cell differentiation, patterning and morphogenesis [220]. But the regulation of animal size remains an area that is still somewhat poorly developed. The control of cell numbers and cell size appears to be the main mechanism by which metazoan organisms regulate their final size [220]. It is a long-standing observation that the size of cells varies according to their biological context but is highly reproducible within the same species, tissue and developmental stage. Understanding how these processes are regulated and coordinated remains an important challenge. In unicellular organisms such as yeast, proliferation rates must be rapidly adapted to nutrient availability, making the coupling of growth and division to nutrient availability a necessity for optimal fitness. In contrast, the goal of developmental programs in metazoan is to tailor proliferation rates so as to ensure appropriate organ and

organismal growth. In this case, cell-to-cell signaling through extracellular factors, rather than nutrient availability, predominantly influences cell growth and division [220].

Here, growth (i.e. addition of cell mass) and cell division or cell cycle are considered as two separate events in the life of the cells. It is still unclear whether these two events are separately demarcated or whether are they strongly interdependent on each other.

In mammalian systems, studies have suggested that Insulin/IGF 1 signalling can stimulate growth and proliferation. Its positive effect on cell cycle is seen in certain but not all cell types. Moreover, the signalling pathways initiated by IGF signalling in various cell types can vary. The discovery of a similar pathway in Drosophila has helped answering the cell cycle question further. The results from mammalian systems and Drosophila system are discussed in detail in Chapter 3.

The Insulin/IGF pathway and longevity

The potential link between aging and insulin/insulin-like growth factor I (IGF 1) signaling has attracted substantial attention on the basis of several pieces of evidence showing that disruption of the insulin/IGF 1 signaling cascade can significantly extend life span in diverse species, including yeast, worms, fruit flies, and rodents. Although the underlying mechanisms of longevity are not fully understood, it is known that mutations in genes encoding components involved in the insulin/IGF 1 signal response pathway can significantly extend life span [221-224]. Examples include *daf-2, age-1,* and *daf-16* mutants of the *Caenorhabditis elegans*, yeast *sch9, and CYR*-null mutants, *InR* and *Chico* homozygous mutant female flies, and long-lived *Prop1*- and *Pit1*- mutant mice [221-225].

Project Overview

After almost 86 years of the discovery of the first molecule of the Insulin Family-Insulin- several research scientists have devoted time and effort to decipher the importance of the members of this family of proteins/hormones in humans and other species. This research has clearly underlined the importance of the role of these ligands, their receptors and their signaling, in fundamental life processes like metabolism and growth. It is now also clear that dysfunction of these processes controlled by insulin family members can lead to several life threatening conditions and hence study of the insulin family and pathways have been very important from therapeutic point of view.

As is evident in the general introduction and other Chapters, most of our knowledge of the fundamental processes of metabolism and growth, and the role of insulin family members in these processes come from mammalian experimental and model systems. Lower model organisms like *Drosophila melanogaster*, *Caenorhabditis elegans* or *Brachydanio rerio*, have only recently been shown to have conserved ligands, receptors and signaling mechanisms. It has become evident that studies in lower organisms can help, in many ways, to add to the knowledge existing from vertebrate studies.

In 2001, our lab identified seven insulin-like genes in Drosophila based on bioinformatics approach. The aim of this Ph.D. project was the biological and chemical characterization of Drosophila Insulin-like Peptides. Main questions and topics addressed during this Ph.D. project can be broadly outlined as follows.

- Comparison of the behavior of the DILPs towards the receptor, comparison of their signaling potencies, check for receptor any agonist activity
- Comparison of the activity of DILPs on growth and proliferation
- Comparative studies of the effect of DILPs on glucose uptake
- Studying the signaling effects of some of the DILPs on various tissues and studying the tissue specific distribution and expression of the Drosophila Insulin receptor

A variety of biochemical, cellular and molecular biological methods were used for the characterization of various Drosophila Insulin-like peptides. These shall be discussed in detail in the following chapters.

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Chapter 2

Synthesis and Purification of Drosophila Insulin-like Peptides 2, 3, 4 and 5

Insulin-like Peptides in Invertebrates (Insects)

The first indications regarding the existence of Insect Insulin molecules date from some 20 years ago [8-10]. Bombyxin, derived from the silkmoth *Bombyx mori,* was the first insect insulin-like molecule to be discovered. It was isolated in 1984 [13] as a brain peptide exerting prothoracicotropic activity in another moth species, *Samia cynthiaricinii.* The structure of Bombyxin was shown to be a heterodimer of (insulin-like) A and B peptide chains which appeared to be linked by means of disulfide bonds in exactly the same manner as in mammalian Insulins, thus forming an Insulin-like core structure [14]. Nevertheless, the organization of the bombyxin gene(s) is markedly different from that of vertebrate insulin genes. The copy number of genes encoding bombyxin-like peptides exceeds 30 per haploid genome of *B. mori.* These genes have been classified into at least 7 subfamilies [14-22] according to their sequence similarities [14-22]. The genes coding for different Bombyxins differ from their vertebrate orthologs in that they lack introns altogether [16, 17]. The general organization of the ancestral gene from which vertebrate and invertebrate insulin superfamily members evolved is probably not conserved in the case of multiple bombyxin genes. Four pairs of median neurosecretory cells are the major site of bombyxin gene expression in the brain [22].

Another insect insulin-like peptide was identified in *Locusta migratoria* [23]. Based on sequence data, Locust Insulin-related peptide (LIRP) did not appear to be much more closely related to bombyxins than to vertebrate insulins or IGFs [24]. However, both bombyxin and LIRP showed a higher degree of sequence similarity to their vertebrate homologs than to the molluskan insulin-like peptide, MIP. A remarkable feature of LIRP is the existence of two transcripts differing in their 5'-UTR. This could be caused by an alternative usage of two different gene promoter situated upstream of the coding region [25]. Whereas *lirp-t1* was only observed in the brain, more specifically in the A2 median neurosecretory cells of the pars intercerebralis*, lirp-t2* was detected in many different tissues, including the brain, the epidermis, the fat body, the midgut, the prothoracic glands, the pterotheca, the thoracic muscle, the ovary, the testis, the follicular cells and the mature oocyte [25].

Recently, the database of the Berkeley Drosophila Genome Project (BDGP) [11] was searched for gene sequences encoding small proteins with insulin-like sequence properties. Advanced BLAST searches with known insulin sequences revealed the existence of several putative insulin-like peptide precursor genes in the Drosophila genome [1, 12]. Five putative peptides, designated as DILP 1–5 (Drosophila insulin like peptides 1–5), are encoded in a *dilp* gene cluster localized on the third chromosome (*dilp 5* is separated from *dilp 4* by one intervening gene). They display in their A and B chains the highly conserved spacing between cysteine residues that is typical for all known members of the insulin superfamily [1, 12]. Two additional genes, *dilp 6* and *dilp 7*, were found to be present on the X-chromosome at two distinct loci [1]. The encoded peptide sequences of the latter each contain a single amino acid insertion between the second and the third cysteine of the putative A-chain (Fig. 1). The predicted organization of these Drosophila insulin-like peptide (DILP 1–7) precursors is very similar to that of mammalian preproinsulin, consisting of a secretory signal peptide, a B-chain, a C-peptide and an A-chain. The length of these putative *Drosophila melanogaster* preproinsulins varies from 107 to 156 amino acids [1].

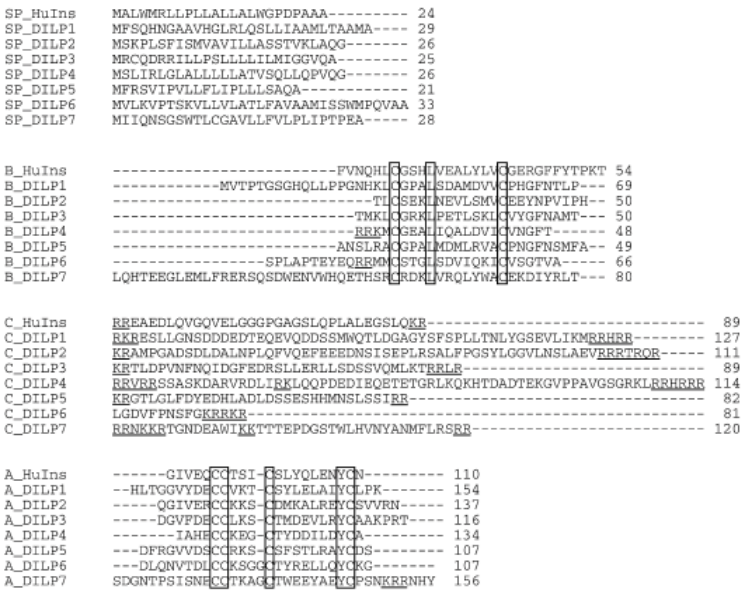
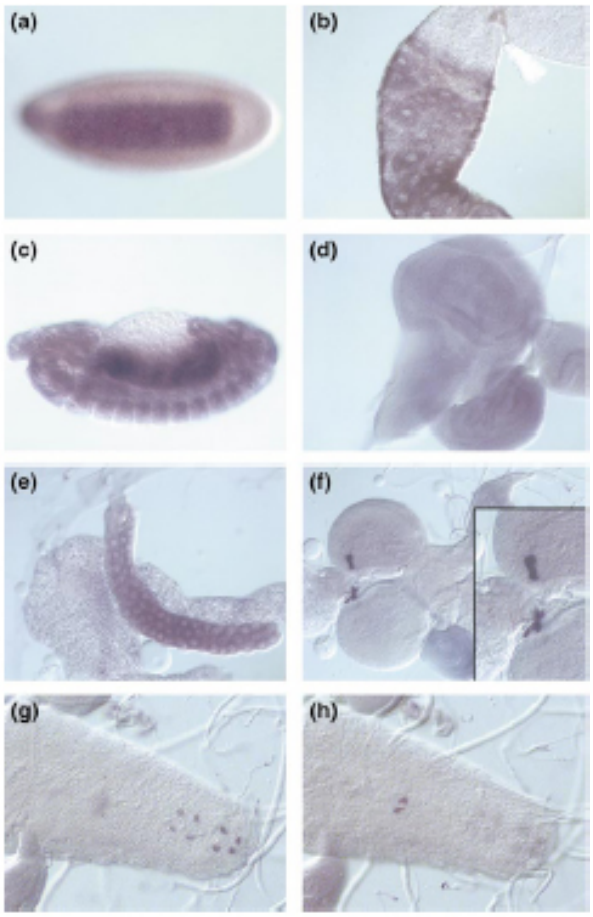


Figure 1 (published by Brogiolo et. al., 2001, Ref [1]) shows Amino acid sequence and alignment of the predicted domains of *Drosophila* insulin-like peptides (DILP1–7) and human preproinsulin (HuIns). Amino acids conserved in all sequences are boxed, including the cysteines that are involved in disulfide bond formation. In DILP1–5, the cysteines retain the same relative position to each other, whereas in DILP6 and DILP7, the A domain (A_DILP) contains four instead of three intervening residues between the second and third cysteine. Optimal predicted cleavage sites containing basic dipeptides are underlined. Predicted cleavage sites occur at the ends of the connecting peptide (C_DILP) between the A and B domains (B_DILP), and are cleaved to give rise to an active peptide. DILP 6 is predicted to

have a short connecting peptide with only one potential cleavage site. DILP 1, 6, and 7 contain an extension at the N terminus of the B chain. In DILP 6, this extension ends with a basic dipeptide liberating a potential short additional domain between the signal peptide (SP_DILP) and B chain. DILP 7 has an extension at the C terminus of the A chain, similar to the D domain of IGFs. It is not clear however, if the N or C terminal extensions of DILP 6 or 7 are cleaved of or are retained in the mature peptide. The six cysteines represent a conserved pattern among all insulin-related peptides. DILP 2 shares maximum homology (52%) with human insulin, but also with IGF 1 (26%) and IGF 2 (28%).



Transcripts of *dilp 2, 3* and *5* are expressed in the median neurosecretory cells (mNSC) in the pars intercerebralis region of the larval and adult Drosophila brain (Figure 2, Published by Brogiolo et. al., 2001, Ref [1]). *dilp 4* mRNA is expressed in the larval midgut and the presumptive mesoderm of the blastoderm stage. *dilp 7* is expressed in a segmental fashion in the ventral nerve cord in four pairs of ventrally located cells in the posterior-most segments and in one pair of dorsally located cells in the abdominal segment A1 or A2. *dilp 1* is probably slightly expressed in the same mNSCs [Rulifson, unpublished data]. Expression pattern for dilp 6 and 7 could not be established through mRNA *in situ* hybridization (Tomoatsu Ikeya, personal communication)

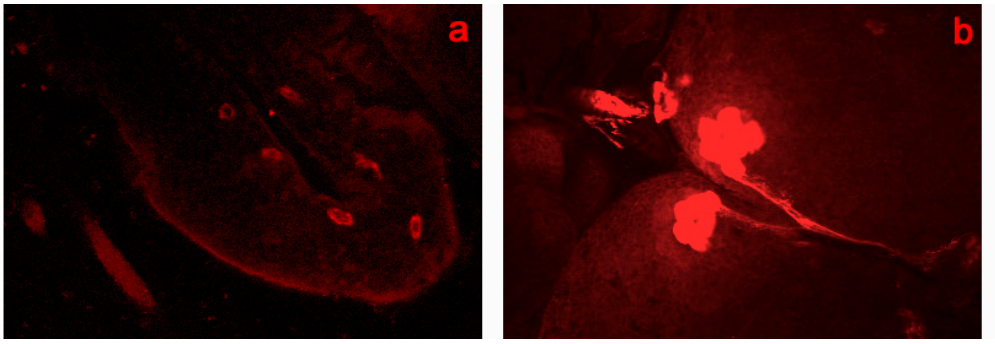


Figure 3: a, 4 pairs of laterally located neurons in Drosophila larval ventral ganglion. The neurons were detected by anti-DILP 7 rabbit polyclonal antibody. Anti-DILP 2 rabbit polyclonal antibody recognises DILP 2 in pairs of seven mNSCs. Axonal projection can be traced to the ring gland and below towards the aorta of the heart.

Insulin-like Peptides in Invertebrates (*Caenorhabditis elegans*)

To identify *C. elegans* insulin super family genes, Pierce et al. [26] analyzed the entire *C. elegans* genomic sequence with multiple sequence searching tools, including BLAST, FASTA, Markoff and the Regular Expression Searches. They examined candidate ORFs encoding at least a putative signal sequence, B chain, and A chain using a stereo chemical restriction model derived from structural elements common to Insulin superfamily proteins as follows: (1) Two helices within the A peptide joined by a loop; (2) an extended amino-terminal coil within the B peptide followed by a tight turn and a central helix; (3) a hydrophobic core that forms the interface between the A and B peptides; and (4) at least three disulfide bonds formed by conserved cysteine residues. This procedure revealed 37 candidate genes, *ins-1* through *ins-37*. In addition to conservation of structural domains, 34 of the 37 *ins* genes have an intron/exon organization identical to most previously described members of the superfamily; the B and A chains are encoded by individual exons separated by an intron at the 3'-end of the B chain coding region.

The *C. elegans* insulin gene family members were classified into four major classes on the basis of predicted arrangement of disulfide bonds [27]. Type-γ insulin-like molecules have display three disulfide bonds as found in vertebrate insulins, IGFs, and most other previously identified members of the superfamily. Type-β proteins contain the three canonical disulfide bonds as well as an additional inter chain disulfide bond between B22, B23, or B24 and A21. The only previously described members of this class are the seven mollusc MIP proteins [27a]. Eight of the type-β *ins* genes encode proteins containing a novel domain between the signal sequence and the B peptide, which is termed the F peptide. Type-α proteins lack the

A6/A11 disulfide bond helices. In *ins-21*, *ins- 22*, and *ins-23*, the cysteines are replaced by amino acids containing aromatic side chains. *ins-31* is a unique member of the gene superfamily; it has a signal sequence followed by three insulin repeats organized in the sequence (B/A)(B/A)(B/A). This gene may encode a single secreted protein containing three insulin-like structural domains or a pre-proprotein that is cleaved into multiple insulin-like peptides. Many members of the *C. elegans ins* gene family are organized into clusters, usually with members of the same class, in which the *ins* genes are tandemly arrayed. Pierce and colleagues show that despite wide variation in amino acid sequence from previously identified insulin-related proteins, INS-1, INS-4, and INS-21 are predicted to adopt the characteristic insulin fold. INS-1 is the most closely related to human insulin by primary sequence comparison, structural homology models, and because it has a probable cleaved C peptide. ins-18, with a C peptide, antagonizes daf-2 signaling. They constructed gene fusions of the upstream promoter and enhancer regions for 15 of the *ins* genes, including *ins-1*, *ins-18*, *ins-9*, and *ins-22*, to the coding region of GFP. The above *ins* genes are expressed primarily in subsets of neurons throughout most of the life cycle. Two of the thirty-seven *C. elegans* insulin superfamily members, INS-1 and INS-18, are predicted to have cleaved C peptides [26].

Although in mammalian systems, the biochemical characteristics of Insulin and Insulin-like ligands and their interaction with the receptors have been studied in some detail, only one biochemical study has been published so far on ligands in invertebrates which focus on the biochemical characterisation of these molecules. They already indicate that detailed biochemical study of this large number of ligands can provide new information to the knowledge that already exists on members of the Insulin family. For example, Weiss et al. [6] have shown that INS-6, a single chain polypeptide expressed in specific neurons of *C. elegans*, despite multiple non-conservative changes in sequence, recapitulates an insulin-like fold. Although lacking classical receptor-binding determinants, INS-6 binds to and activates the human insulin receptor. Its activity is greater than that of an analogous single-chain human Insulin. To pursue such studies, the group synthesized INS-6 chemically and purified it.

Thus, in order to pursue biochemical characterization of the Drosophila Insulins, I decided to synthesize and purify the DILPs. Out of the seven DILPs, I could successfully synthesize DILP 2, 3, 4 and 5. The other DILPs could not be purified due to technical constraints as discussed later. The DILPs were purified by following methods which are used routinely for the synthesis of human Insulin. The procedures are described in detail under 'Materials and Methods' and are discussed in the Results and Discussion section. Once the DILPs were

purified, their activities were tested and compared. The binding affinities towards the receptor were compared by competitive binding assay.

Materials and Methods

Anti-DILP polyclonal Antibodies

Polyclonal rabbit antibodies were generated against short peptides in the DILPs. The peptide sequences are as follows

dilp 1: -NH_2-HQLLPPGNHKLC-$CONH_2$

dilp 2: -NH_2-CEEYNPVIPH-$CONH_2$

dilp 3: -NH_2-CGRKLPETLSKL-$CONH_2$

dilp 4: -NH_2-CTYDDILDY-$CONH_2$

dilp 5: -NH_2-CGPALMDMLRVA-$CONH_2$

dilp 6: -NH_2-CTYRELLQY-$CONH_2$

dilp 7: -NH_2-RSQSDWENVWHQETHS-$CONH_2$

The peptides were chemically synthesized and injected into Rabbit in 3 booster doses. First two bleed serums were checked for the presence of antibodies. After 3rd boost the rabbits were killed and serum was collected. The serums were affinity purified to enrich IgG and IgA fractions. For immunostainings the antibodies were used at 1:1000 concentrations in PBT (0.2% BSA, 0.01% Tween-20 in PBS). Secondary antibodies were used at 1:500 concentrations.

Expression of recombinant peptide chains

DILP 2 cDNA corresponding to A and B chains of the putative protein was amplified by PCR from a pool of cDNA obtained from third instar larvae (Qiagen Omniscript RT kit, Catalog no- 205110). The PCR fragments cloned into TOPO TA Cloning® vector (Invitrogen, Catalog No-KNM4500-40) and cleaved using Eco RI / BamHI sites. The fragments were cloned into the pET-32a(+) expression vector (Novagen, Catalog no- 69015). The expression vector was linearized using the same enzymes so that the thrombin cleavage site was deleted from it. Instead of using the regular Thrombin cleavage site of the vector, an Enterokinase cleavage site between the peptide chain sequence and the C- terminal tag was inserted. Primer sequences are as follows: dilp2 A chain forward primer

ACTAGATCTTGACGACGACGACAAGGCAGCACCTCTCCACGATTCCTTG

dilp2 A chain reverse primer

ACGGAATTCCTAATTTCTGACCACGGAGCAGTAC
dilp2 B chain forward primer
ACTAGATCTTGACGACGACGACAAGACGCTCTGCAGTGAAAAGCTCAACG
dilp2 B chain reverse primer
ACGGAATTCCTAGTGGAATCACGGGATTATACTCCTCG.

The reading frame was verified by sequencing. The vectors were transformed into BL21(DE3) strain of bacteria (Novagen, Catalog no- 69387) using 1-2 ug DNA per 100 ul of competent cells. Single ampicillin resistant colonies were picked and used to inoculate a 5ml overnight 'starter culture' using 2x YT medium containing 0.1 µg/µl Ampicillin. 500ml of fresh 2x TY medium was inoculated with 500ul of starter culture and shaked in a rotary shaker at 37° till an OD of 0.4-0.6 was reached. To start protein expression, the culture was then induced with IPTG at a final concentration of 1mM. The culture was shaked at 30° for two hours and then placed on ice. All further steps were carried out at 4°C. Bacteria were collected by centrifugation at 13,000 rpm at 4° for 13 minutes (Sorvall RC-58). The pellet was washed with cold loading buffer (for His bind column, 20mM Na_2PO_4, 0.5mM NaCl, 20mM Imidazole, pH 7.4) and resuspended in this buffer. The solution was sonicated on ice for 10 min at high frequency to lyse the bacteria and extract soluble proteins. The lysate was centrifuged at 13,000 rpm at 4° for 13 minutes. The supernatant obtained was checked on an SDS PAGE gel for the presence of expressed protein (Figure 4a).

Purification of recombinant protein

To purify the 6x His tagged protein, the supernatant lysate was passed through a His-Trap HP 1ml column (Amersham, catalog no- 17-5247-01). The tagged protein was eluted using His column elution buffer (20mM Na_2PO_4, 0.5mM NaCl, 500mM Imidazole, pH 7.4). The eluate fractions were checked for purified protein. Eluate fractions containing the expressed protein (checked with SDS PAGE and comassie staining) were passed through 5ml HiTrap Desalting columns (Amersham, catalog no.- 17-1408-01) to 'desalt' (remove Imidazole). The tagged protein was now in Enterokinase tag cleavage buffer (50mM Tris HCl, 10mM $CaCl_2$, 1% Tween-20, pH 8.0). Final concentration of tagged protein was measured by comparison with BSA standards on an SDS PAGE and reconfirmed by Dc Protein assay (Biorad, catalog no- 500-014).

Tag Cleavage and purification of peptide chains

Once concentration and purity of the tagged protein was established, the tag was cleaved using EKMax™ Enterokinase (Catalog no.- E180-01). Each unit of EKMax™ was used to cleave 20ug of tagged protein overnight at room temperature. Control was run in parallel using sample without Enterokinase. The reaction mixture was fed onto a Reverse Phase HPLC column (Macherey and Nagel, Catalog no.- 715002, Standard-Prep HPLC column SP 250/10 NUCLEOSIL 100-7 C_{18} length: 250 mm, ID: 6 mm). Proteins were eluted in isochratic gradient using 0-100% Acetonitrile (HPLC grade, Fluka, catalog no.- 00683) containing 0.01% Tetra flouroacetic acid (Pierce, catalog no.- 28902) in 50 minutes. The same gradient conditions were used for all the following RP HPLC runs. Various peaks were collected and the eluate containing acetonitrile and water was dried in a vaccum dryer. Mass of various peaks was identified using mass spectrometry (by Protein Analysis Unit, University of Zurich).

Chemical synthesis of peptide chains (by Dr. Bhoopathy Dhanapal, Lipal Biochemicals)

To begin each coupling, the Fmoc group on the resin bound amino acid/peptide was removed with 20% piperidine in N,N-dimethyl formamide (DMF). It was then rinsed and a protected amino acid was added which has been activated at its 'alpha' carboxyl group. The activation was achieved by creating the N-hydroxybenzotriazole (HOBt) ester *in situ*. The activated amino acid and the resin bound amino acid were allowed to react in the presence of base to form a new peptide bond. This process is repeated until the desired peptide is assembled at the resin. Once the peptide is complete, it is ready to be cleaved from the resin. This is accomplished using a mixture of trifluoroacetic acid (TFA) and scavengers. Scavengers serve to neutralize cations which are formed during the removal of the side chain protecting groups. The solution is at least 82% TFA, and the rest a mixture of phenol, thioanisol, water, ethanedithiol (EDT), and triisopropylsilane (TIS). The peptide on the resin is allowed to react with the cleavage mixture for several hours, which then affords the peptide in solution. It can then be precipitated and washed in *tert*-butyl methyl ether, and analyzed or purified as desired by RP-HPLC. Before use *N*,*N*-dimethylformamide (DMF) was dried ($MgSO_4$) and redistilled under reduced pressure from ninhydrin. *N*,*N*-Diisopropylethylamine (DIEA) was redistilled first from ninhydrin then from KOH. The monitoring of the deprotection was performed by removing a aliquot of the 20 % piperidine / 80 % NMP deprotection solution after 1.75 min. This sample was passed through the UV detector at 301 nm where a peak was recorded. After washing with NMP the deprotection step was repeated and a second peak was recorded. If the height of the second peak was less than 4 % of the first peak the deprotection was continued

for 8 more minutes before the rest of the coupling cycle was carried out. If this condition was not met, the deprotection step was performed a third time and monitored (duration 10 min). These deprotections were repeated until the condition was met.

Starting material were from Fluka, Advanced ChemTech, Novabiochem or PerSeptive Biosystems. Fmoc-protected amino acids and resins were purchased from Fluka, Advanced ChemTech, Novabiochem, Bachem or Rapp Polymere. For purification and analysis reversed-phase high performance liquid chromatography (RP-HPLC) was performed using either a dual pump *LKB* Bromma (Pharmacia), a single pump HP 1050 series or a dual pump Äkta purifier (Amersham Pharmacia Biotech) system. The columns used were: Waters RCM αBondapak™ C_{18} catridges (10 m, 125 Å, 8 x 100 mm, flow rate: 2 ml/min), Vydac™ C_{18} 218TP104 (10 αm, 125 Å, 4 x 100 mm, flow rate: 1 ml/min) and Vydac™ C_4 214TP104 (10 αm, 125 Å, 4 x 100 mm, flow rate: 1 ml/min) for analytical separations. Waters RCM- α Bondapak™ C_{18} catridges (10 α m, 125 Å, 25 x 100 mm, flow rate: 8 ml/min), Vydac™ C_{18} 218TP1010 (10 αm, 125 Å, 10 x 100 mm, flow rate: 5ml/min) and Vydac™ C_4 214TP1010 (10 αm, 125 Å, 10 x 100 mm, flow rate: 5 ml/min) for preparative separations. UV-detection was at α= 226 nm and 278 nm, or just 226 nm for HP1050 series. Solvents used were acetonitrile from Biosolve and dist. water, each containing 0.1 % trifluoroacetic acid (TFA).

Sulphonation of the chains

In several cases the peptide chains were sulphonated. For sulphonation of the peptide chains (synthetic or recombinant), for each mole Cysteine in the peptide chains 100x moles of Sodium sulphite, Na_2SO_3 (Sigma, catalog no.- S4672), and 10x moles of Sodium tetrathionate, $Na_2S_4O_6$ (Fluka, catalog no.- 72028) were used. The dried peptide chain was resuspended in 8 M Guanidine chlorides, 0.1M Tris HCl, pH 8.8 also containing the above two salts. The reaction was stirred vigorously in an open glass vial for 3 hours at room temperature. The resulting solution was dialyzed overnight with water using Spectra/Por® Float-A-Lyzer® (Spectrum Labs, MWCO 500, Catalog no. 235036). The resulting dialysate was carefully collected and fed onto RP HPLC column, and the peak of the sulphonated peptide was collected with a dropper. The concentration of the sulphonated peptide obtained was measured by using Bradford assay.

Ligation of sulphonated peptides

For ligation of the sulphonated A and B peptide chains, published procedures were followed. The sulphonated peptides were mixed in the ratio of 2A:B. The reaction was performed in a

0.1M Glycine buffer. Two mole of DTT (Fluka, for molecular biology, Catalog no. 43815) per mole of B chain (containing two cysteines) plus four moles of DTT per mole of A chain (containing four cysteines) was added. The final pH of the solution was adjusted to 10.6. The solution was shaked in an open wide-mouthed vial overnight at 4°C. The reaction mixture was fed onto an RP-HPLC column and various peaks were collected. The peak of DILP was identified my mass spectrometry. The final amount of DILP obtained was measured again using spectrophotometry (NanoDrop, ND1000) and confirmed by Bradford assay.

Ligation of unsulphonated peptides

For ligation of unsulphonated A and B peptide chains the peptides were mixed in the ratio of 2A:B in 0.1M Glycine buffer and adjusted to pH 10.6 (no DTT added). The solution was shaken in an open wide mouthed vial overnight at 4°C. The reaction mixture was fed onto an RP-HPLC column and various peaks were collected. The peak of DILP was identified my mass spectrometry. The final amount of DILP obtained was measured using Spectrophotometry (NanoDrop, ND1000) and confirmed by Bradford assay.

Repurification of the peaks and analytical HPLC

The peaks of DILPs obtained above were run once again on a preparative RP-HPLC column (described above) to check the purity of the DILP obtained. The whole amount of DILP was dissolved in optimal quantity of water and fed onto a preparative column. The prominent peak was collected again, so as to separate other smaller peaks (corresponding to other impurities). Once the peak was repurified, a small amount of DILP was run on an analytical column (Macherey and Nagel, Nucleosil® Standard RP, C18 column) again to check its purity. The gradient conditions of all the runs were the same as described above.

Determination of DILP activity

Activity tests of various DILPs were done using Drosophila cells in culture. Kc 167 cells were grown to 60-80% confluence in Schneider's medium (Invitrogen, catalog no. - 11720-034) with 5% fetal calf serum (Invitrogen, catalog no.- 16000-044) and penicillin/streptomycin. DILPs were added to various concentrations (0.1nM-1μM) whereas non stimulated cells were used as controls. Insulins were added to achieve different final concentrations ranging from 0.1 – 100 nM. After 20 min. stimulation the cells were placed on ice and collected by centrifuging at 300xg for 5 minutes at 4°C. The supernatant was discarded and the pellet was washed by the cold PBS. Cells were lysed by lysis buffer (120mM NaCl, 50mM Tris-HCl,

20mM NaF, 1mM Benzamidine, 1mM EDTA, 6mM EGTA, 15mM $Na_4P_2O_7$, 1% Nonidet P-40) containing protease inhibitors like 1x Mini Cocktail Tablets (Roche, Catalog No. 04 693 159 001) and phosphatase substrates like pNPP (Fluka, Catalog no.- 71770) and β-Glycerol phosphate (Sigma, Catalog no.-G6251). The cells were shaken in lysis buffer at 4°C for 15 minutes, and the lysate was centrifuged at 4°C at 13,000 rpm for 15 min. The supernatant was collected and total protein concentration was estimated. 100 µg of total protein of lysate was heated with 4x Loading buffer (0.5M Tris HCl, 5mM DTT, 2% SDS, 1% Glycerol, 0.02% Bromophenolblue) and loaded on a 10% SDS PAGE gel. The proteins on gel were blotted on a Nitrocellulose (Amersham, Hybond™ ECL™, catalog no.- RPN2020D) membrane overnight at 4°C at 150mA. Thereafter the membrane was blocked with 3% ECL blocking solution (Amersham, catalog no.- RPN418). Primary antibodies like anti-phospho PKB (Ser 473) (Cell Signaling, catalog no.- 9271S), anti-phospho p70-S6 kinase (Thr 389) (Cell Signaling, catalog no.- 9205S) and anti-tubulin (Sigma, catalog no- T9026) were used at 1:1000 (phospho-specific antibodies) and 1:100,000 (anti-tubulin) respectively. Secondary antibodies like anti-rabbit HRP conjugated secondary antibody (Jackson Immunoresearch, catalog no.- 111-035-003) and anti- mouse HRP (Amersham, NA931VS) were used at 1:10,000 dilution. The blots were developed using ECL Western Chemiluminescence Kit (Amersham, catalog no.- RPN2109).

DILP activity on tissues

To check the activity of DILPs on Drosophila tissues, flies expressing a tGPH reporter trangene, were used [described in 50]. First-second instar larvae expressing tGPH reporter were picked and grown on 20% sucrose solution. On the fifth day after egg laying, the larval fat body was isolated in PBS. DILP 2, 3, 4 or DILP 5 were added to a final concentration of 100nM in PBS and left covered for 45 minutes at room temperature. The fat bodies were then fixed with 4% paraformaldehyde, washed and mounted. The samples were photographed for GFP using a Confocal microscope.

DILP competitive binding assay

Kc cells were plated at 3 x 10^6 cells/ well in six well plates, 48 hours before each experiment and fresh medium was given 24 h before the experiment. Cells were washed twice with PBS, and then 1 ml of cold binding buffer (120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 9.0 mM glucose,100 mM HEPES, 1% BSA, pH 8.0) containing 0.1nM I^{125}-Human insulin was added to each well. The plates were then incubated at 4 °C for one hour. This time was sufficient to achieve >90% of maximal binding. Various concentrations of DILP 2 and DILP 3 (0.1nM,

1nM, 10nM, 100nM, 1μM) were added and plates were incubated at 4°C for another one hour. The medium was removed and cells were washed three times with ice-cold PBS and solubilized with 0.1% SDS. Aliquots of the lysate were counted for bound radioactivity. Values obtained when 1.5 μM unlabeled human insulin was present during the binding reaction were subtracted from the total radioactivity to give specific binding. Cells were counted in control wells, and the binding was normalized to 10^6 cells. Normalized counts were plotted against the concentration of DILP 2 or 3 used.

Results and Discussion

One of the most important aspects of purification of any functional protein is the formation of correct bond formation to give the correct three dimensional structures. Insulin is a small molecule with its hallmark being the intra-chain and inter-chain disulphide bonds between its two peptide (A and B) chains. Hence, for purification of active insulin, it is important to maintain correct bond formation and correct folding of the two chains.

Insulin is synthesized in humans and other mammals within the β-cells of the Islets of Langerhans in the pancreas. One to three million islets of Langerhans (pancreatic islets) form the endocrine part of the pancreas, which is primarily an exocrine gland. The endocrine part accounts for only 2% of the total mass of the pancreas. Within the Islets of Langerhans, β-cells constitute 60–80% of all the cells.

Insulin is translated in the β-cells, in the form of pre proinsulin, which consists of a signal peptide (first 24 amino acids), and a peptide chain consisting of A and B peptides joined together by the C- peptide chain. The signal peptide facilitates the entry of proinsulin into appropriate Golgi vesicles via the Endoplasmic reticulum. Once proinsulin is segregated into special vesicles, the processing of proinsulin to insulin is carried out in two enzymatic steps [28]. First, the proinsulin sequence B chain- Arg-Arg- C chain -Lys-Arg- A chain is cleaved endo-proteolytically at the two dibasic pairs by the prohormone convertases PC2 and PC3 (also called PC1), both of which belong to a family of yeast Kex2-type endoproteases [29, 30]. Secondly, the carboxy terminal basic amino acids are removed exoproteolytically from the newly exposed B-chain of insulin and C peptide by carboxypeptidease H (CPH) [31, 32]. Only specialized cells containing these cleavage enzymes are able to process proinsulin correctly. Attempts have been made to make certain non-pancreatic cell types process proinsulin [33]. Some non-pancreatic cells are able to process proinsulin by co-overexpression of the processing enzymes [34]. Although prohormone convertases have been

reported in Drosophila [35], whether the available Drosophila or any insect lines express these enzymes and whether proinsulin-like molecules can be correctly processed in these cells lines has not been established. Since preliminary experiments did not show expression of mature DILP 2 in Kc cells, the approach (discussed above) of purifying DILPs was ruled out. Also, large scale isolation of DILPs from flies did not seem feasible due to small size of the animals and small amount of DILPs produced and secreted.

One of the two methods that has been widely used for the purification and production of active human insulin is known as the 'one chain method'. This approach involves the expression of correctly folded proinsulin in bacteria, its isolation and then treatment with enzymes to cleave the C chain off [36]. To purify the expressed protein in bacteria, a suitable carrier tag has been used, most common being the β-GAL tag. For large scale productions, the tag is separated from proinsulin by use of cyanogen bromide which cleaves after the amino acid Methionine. Methionine is introduced between the tag and proinsulin chain, thus saving on expensive tag cleaving enzymes. Proinsulin is then treated *in vitro* with trypsin and carboxypeptidase B to cleave off the C-peptide and to isolate the correctly folded insulin [36, 37]. This approach was not possible for the purification of DILPs since many of the proDILPs have Methionine at several positions which would serve as additional cleavage sites for Cyanogen bromide. Second reason for not using this method is that trypsin cleavage sites XR-RX or XR-KX are also present on several locations on the A and B chains of the DILPs. Hence, enzyme cleavage of C-chain would not work correctly.

The second method regularly used for the production of active insulin is known as the 'two chain method'. This method involves the expression of tagged A and B chains separately in bacteria or synthesizing them chemically. The tag is removed either by cyanogen bromide treatment or other means. Then the individually isolated A and B chains are combined under suitable conditions *in vitro* and correctly formed active Insulin is isolated, which is shown to be the predominant product formed. This method seemed feasible for the purification of the DILPs and hence it was followed.

Purification of DILP 2 and DILP 3

The peptide chains used for producing recombinant DILP 2 were obtained from bacteria, according to the procedure described in Materials and Methods. In short, the two peptide chains of DILP 2 were expressed in bacteria along with a tag which contained 6xHis tag. The 6xHis tag was used to purify the protein using a Ni-chelating Histidine binding columns (Fig 4b). The yield of tagged protein obtained was around 2-3 mg of tagged protein per 250ml of

bacterial culture. Fig 4a shows expression of tagged A and B chains of DILP 2 in comparison to non-induced controls. Expressed protein could be easily recognized with prominent bands.

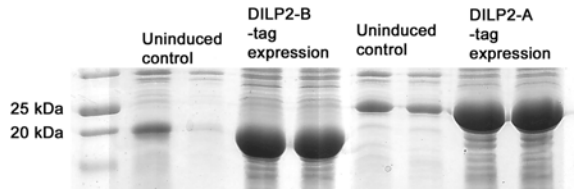


Figure 4a shows comassie stained SDS-PAGE gel showing expression of DILP 2 A and B peptide chains with a protein tag. Lanes (from left) 2, 3 and 4, 5 show lysates from bacteria carrying empty expression vector whereas lanes 4, 5 and 6, 7 show recombinant proteins expressed by bacteria when induced.

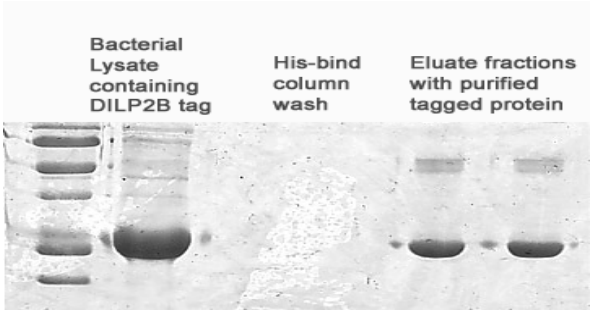
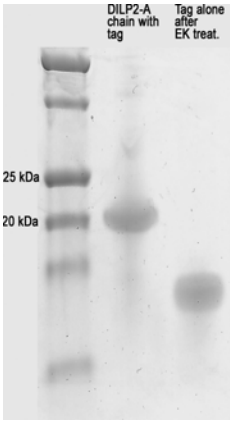


Figure 4b shows purification of the recombinant, tagged DILP 2 B chain by using His-bind columns as seen on SDS-PAGE gel after comassie staining. Lane 1(from left) is Marker, lane 2 shows bacterial lysate showing expressed protein, lane 3 and 4 show column washes and lanes 5 and 6 are His-bind column eluate fractions containing the tagged protein.



The Enterokinase cleavage site between the tag and the peptide chain was used to cleave the tag off. EK cleaves exactly after its recognition sequence (-DDDDK-), thus adding no extra amino acid to the peptide, which might affect the function the DILP 2 later. Figure 4c shows comassie stained SDS-PAGE gel, Lane 1 (from left) is Marker, lane 2 shows DILP 2 A chain tagged recombinant protein, lane 2 shows tag alone after cleavage of the recombinant protein by Enterokinase.

The peptide chain could not be visualized by comassie staining probably due to lower molecular weight and lower quantity. The peptide was identified and purified using RP-HPLC (Figure 4d) and mass spectrometry (Figure 4e).

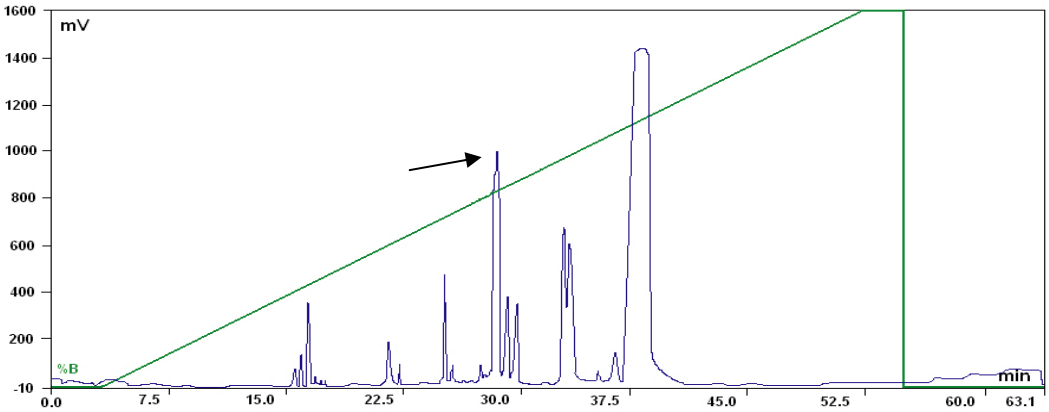


Figure 4d shows EK cleavage reaction products as separated on RP HPLC column. Marked peak is of DILP 2 B chain.

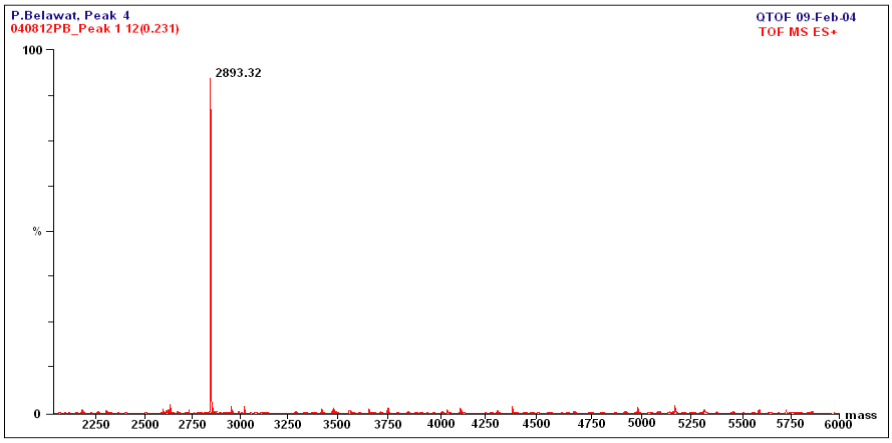


Figure 4d shows Mass spectrometry analysis of recombinant DILP 2 B chain obtained from RP-HPLC

Total yield of the peptide chain (without tag) was around 1mg per 250ml of bacterial culture. Mass spectrometry data showed that peptide obtained from bacteria was completely oxidized, i.e., the disulphide bonds between Cysteines were already formed. To break these preexisting bonds, and make the Sulphur atoms on Cysteines free, the pre- existing bonds are lysed by DTT. To protect the free Sulphurs on these peptide chains from oxidizing again, the free sulphur atoms on Cysteines are sulphonated [38, 39, 40], as shown schematically below

R-S-S-R'----------sulphonation----------$R\text{-}S\text{-}SO_3^-$ + $R'\text{-}S\text{-}SO_3^-$

More than 90% of the peptide chain was converted into its sulphonated derivative and the yield was around 900 µg per 250ml starting culture. The elution time of the sulphonated peptides was shifted by 1-2 minutes as compared to the oxidized DILP 2 B chain. DILP 2 A chain was obtained similarly. The ligation of sulphonated derivatives was done *in vitro*. An important ingredient in this single-step, single-solution combination reaction is the thiol

reducing agent DTT. The chains were mixed in the ratio of 2A chain (4 S-SO3$^-$ bonds):1B chain (2 S-SO3$^-$ bonds). 6x moles (= 6x S-SO3$^-$ bonds) DTT was added to the reaction mixture. The reaction was performed in a small volume (500 µl when combining 400 µg of sulphonated A chain + 200 µg of sulphonated B chain) as published [38, 41]. The DTT is responsible for sulphitolysis (breaking of S-SO3$^-$ bond) and producing free sulphurs on the A and B chains [42]. Hence, it is important to have the exact amount of DTT equal to the bonds, since excess DTT would break new bonds between A and B chains formed. The whole reaction mixture was analyzed on an RP-HPLC column. Correctly folded DILP 2 was the predominant species formed (Fig 5a). The peak of DILP 2 was confirmed by mass spectrometry (Fig 5b). The yield of DILP 2 was around 50% of the B-chain used.

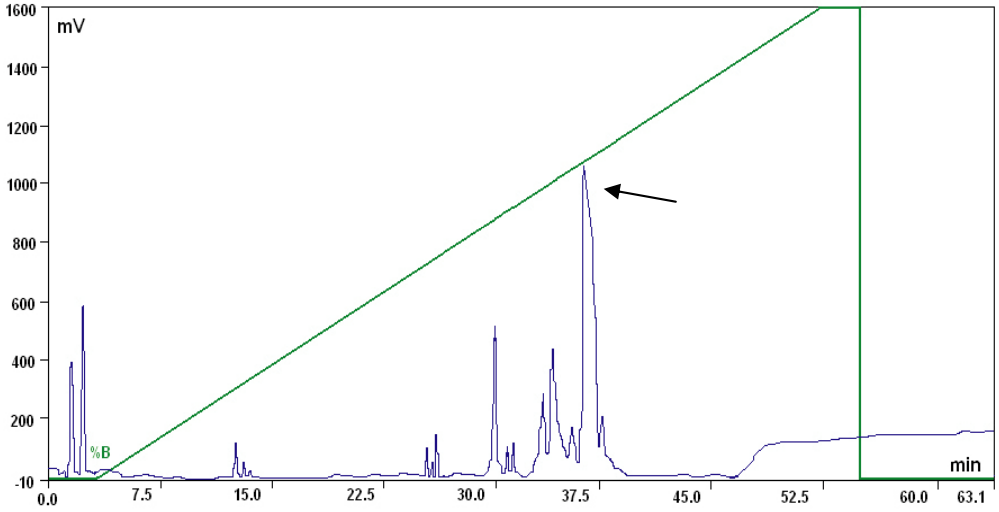


Figure 5a - DILP 2, A and B chain combination reaction as seen on RP HPLC column. Peak of DILP 2 is marked by an arrow

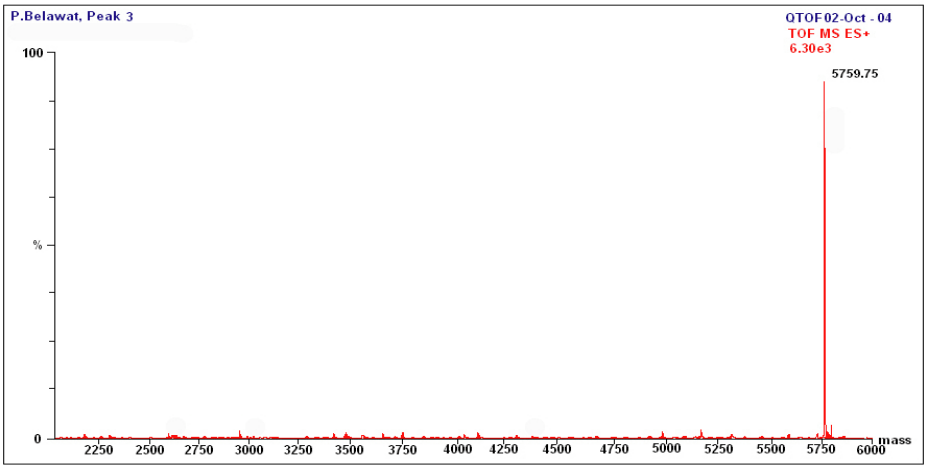


Figure 5b - Mass spectrometry analysis of peak of DILP 2 as obtained from RP-HPLC run

An extraordinary variety of products may result from the chemical combination of the two Insulin polypeptide chains [38, 43]. In fact, the chromatogram displays considerable heterogeneity (Fig 5a). However high yield of DILP 2 shows clearly that the combination reaction does not proceed in a totally random fashion. Some of the factors leading to a high yield of insulin through the chain combination method include specific, sequence governed side chain interactions, facile disulphide interchanges, and the apparent conformational stability of the Insulin molecule [43-46].

The peptide chains for the 'chain combination' method described above can be obtained either through recombinant means (as described above) or by chemical synthesis. We obtained chemically synthesized A and B peptide chains from Lipal Biochemicals. The chemically synthesized chains were up to 90% in the reduced state (peptide report, data not shown), but the synthetic chains were still sulphonated using the procedure described above. The rest of the chain combination procedure for the synthetic chains was as described above. The properties and activity of synthetic and recombinant DILP 2 were indistinguishable from recombinant peptides. Human insulin has been made by using chemically synthesized peptide chains and was found to show identical activity as recombinant human insulin [47-49].

For purification of DILP 3, the same protocol as for DILP 2 was followed. DILP 3 was made and purified using synthetic peptides only. Since the activity of synthetic and recombinant DILP 2 did not show any difference (as discussed later), it was therefore decided to use synthetic peptides for production of other DILPs. Fig 6a and 6b shows the chain combination profile of DILP 3 on an RP-HPLC column and a mass spectrometric identification of DILP 3 peak, respectively. The yield of DILP 3 was significantly lower than that of DILP 2; it was around 35% of the B chain used.

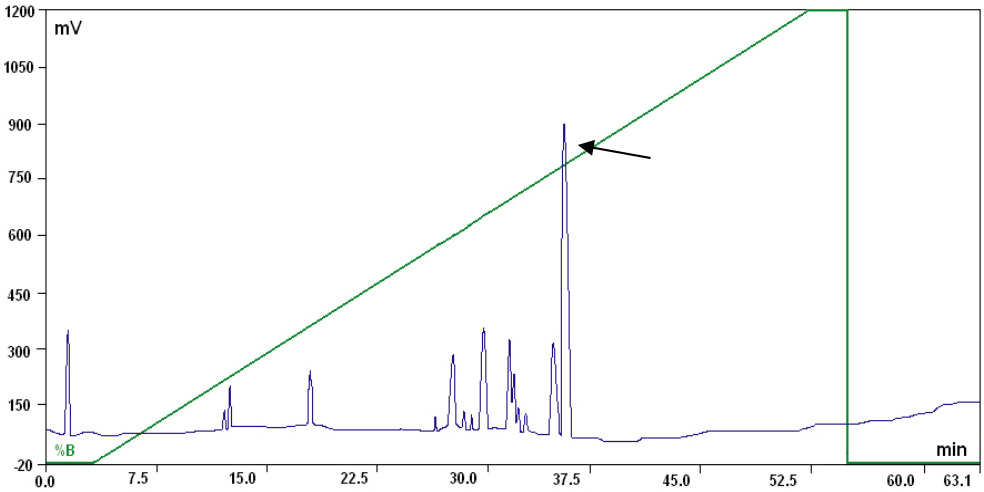


Figure 6a - DILP 3, A and B chain combination reaction as seen on RP HPLC column. Peak of DILP 3 is marked by an arrow

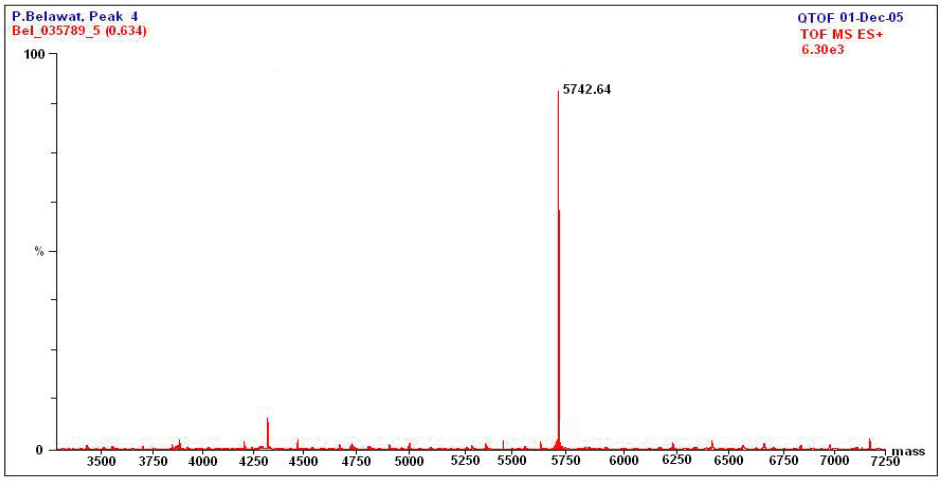


Figure 6b - Mass spectrometry analysis of peak of DILP 3 obtained from RP-HPLC run

Purification of DILP 4 and DILP 5

For the purification of DILP 4 and DILP 5, unsulphonated synthetic chains were used, since the peptides chains proved difficult to be sulphonated and dialyzed. The sulphonated chains were lost during dialysis; probably due to increased affinity to the dialysis membrane. Hence, these peptide chain were used directly (unsulphonated) for ligation (Fig. 7 a, b and Fig 8 a, b). The yield of DILP 4 and DILP 5 was around 20% of the B chain used.

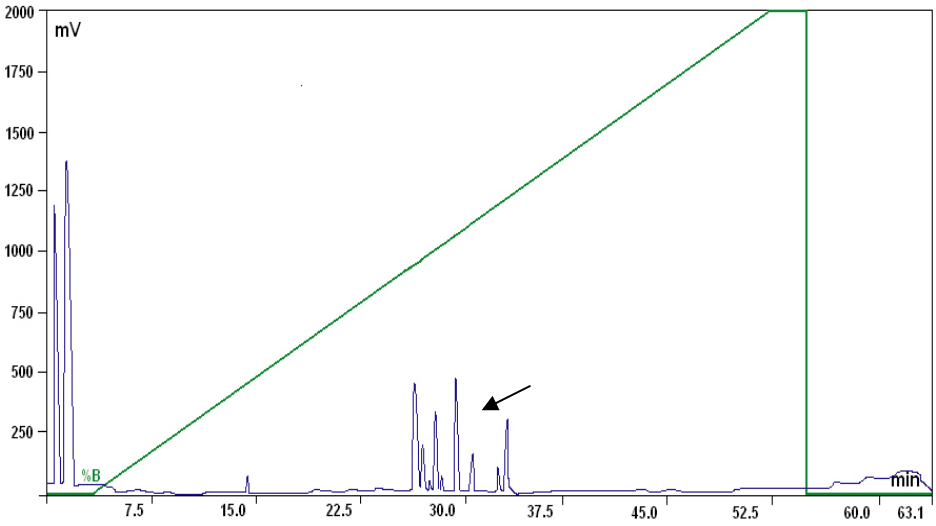


Figure 7a - DILP 4, A and B chain combination reaction as seen on the RP-HPLC column. Peak of DILP 4 is marked by an arrow

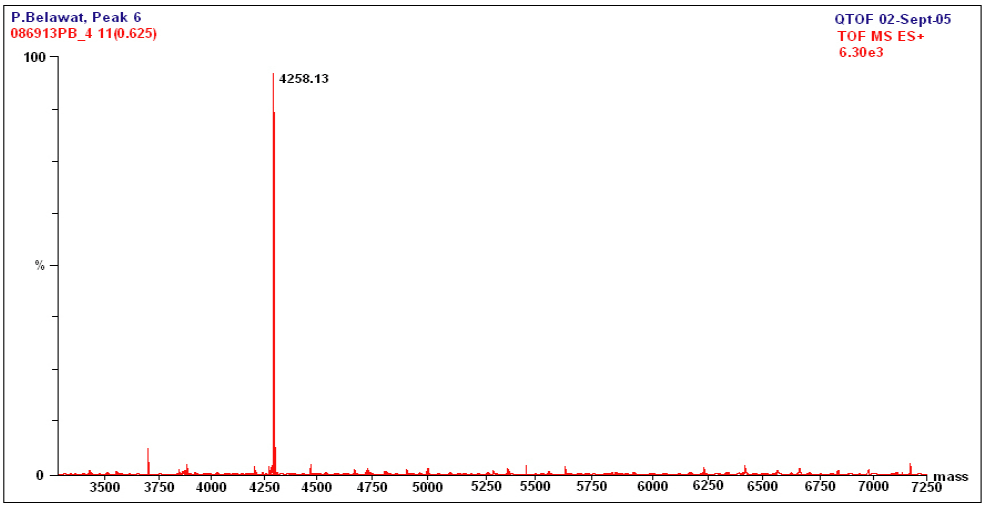


Figure 7b - Mass spectrometry analysis of DILP 4 obtained from RP-HPLC run

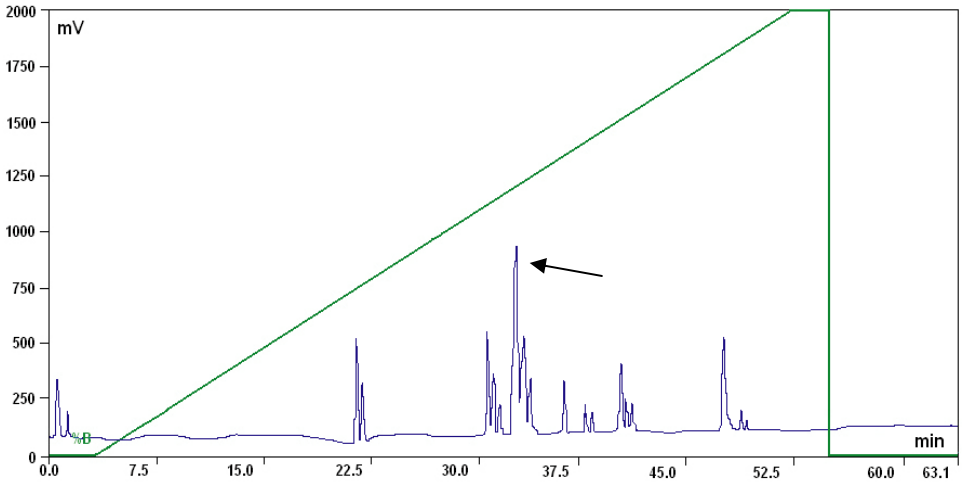


Figure 8a - DILP 5, A and B chain combination reaction as seen on RP-HPLC column. Peak of DILP 5 marked by an arrow

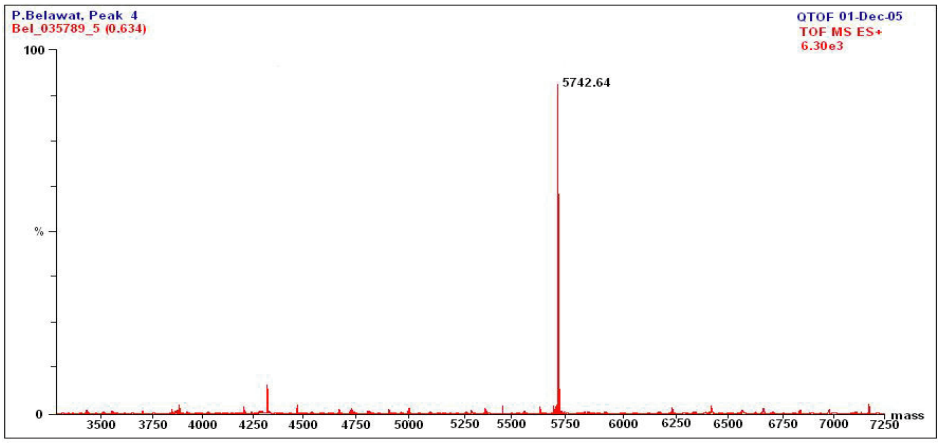


Figure 8b - Mass spectrometry analysis of DILP 5 obtained from RP-HPLC run

All DILPs were re-purified by preparatory RP-HPLC and analyzed by analytical RP-HPLC. All purified DILPs eluted as one single peak on analytical column (data not shown). Mass spectrometric data also confirmed the purity of the DILPs obtained.

DILP 1, DILP 6 and DILP 7

The chemical synthesis of peptide chains of DILP 1, DILP 6 and DILP 7 were predicted to be technically difficult, since atleast one of the peptide chains of these DILPs was too long (more than 35 amino acids). Hence, these DILPs could not be synthesized.

Activity of the DILPs

PKB and S6 Kinase are two main phosphorylation targets of Insulin signaling. The phosphorylation of these two kinases was used as readout to detect and compare DILP activity. Specific serine and threonine phosphorylations on these kinases were detected using phospho- specific antibodies. The phosphorylation was detected by Western blotting (procedure described in Materials and Methods). Activities of DILP 2, 3, 4 and 5 were determined using various concentrations of the ligands (from 0.1nM-1μM). Fig 9a shows that DILP2 can stimulate the phosphorylation of both dS6 kinase and dPKB, at concentrations as low as 1nM, in both S2 and Kc cells. The phosphorylation seems stronger on Kc cells as compared to S2 cells (Fig 9a). Fig 9b shows phosphorylation of S6 kinase and dPKB in Kc cells in response to DILP 2, DILP 3 and bovine insulin. DILP 2 reaches maximal phosphorylation at a concentration of 1nM; whereas DILP3 reaches maximal phosphorylation between 5-10nM and bovine insulin can activate maximally at more than 100nM concentration. DILP 4 and DILP 5 also show maximal phosphorylation at around 100nM concentration (Fig 9c). Hence, DILP 2 is a more potent activator of the pathway as compared DILP 3, 4 and 5. According to their activities the DILPs can be arranged in the following order DILP2 > DILP3 > DILP4 ≥ DILP5.

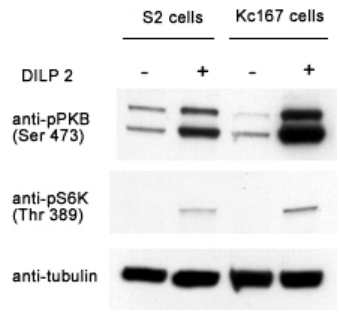


Figure 9a shows phosphorylation of dPKB and dS6 kinase in response to DILP 2, in Kc and S2 cells, as detected by phospho specific antibodies.

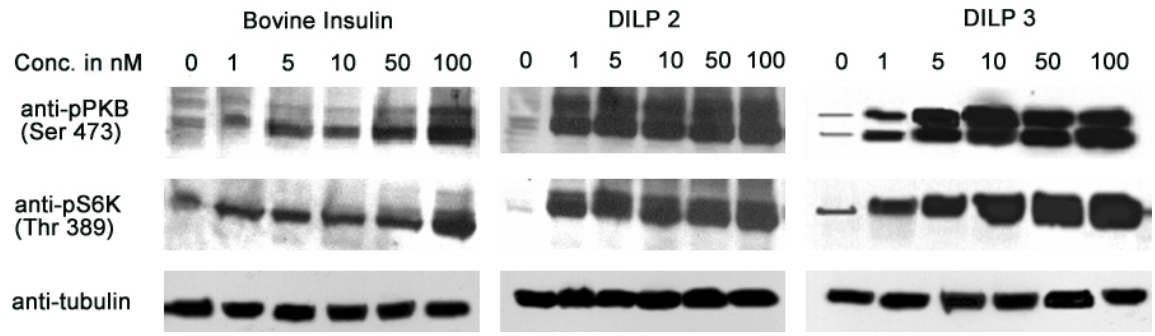


Figure 9b shows phosphorylation of dPKB and dS6 kinase in response to DILP 2, DILP 3 and Bovine Insulin, each tested at various concentrations (from 0-100nM) in Kc and S2. The phosphorylation of the downstream kinases was detected by phospho specific antibodies.

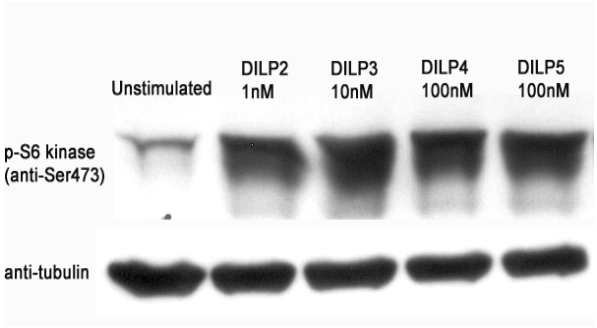


Figure 9c shows comparative phosphorylation of dS6 kinase upon stimulation by DILP 2-5. Comparable phosphorylation of S6 kinase is achieved by using 1nM DILP 2, 10nM DILP 3, or 100nM DILP 4 or 100nM DILP 5 are used. The phosphorylation of dS6 kinase was detected by phospho specific antibodies.

One possible explanation of the difference in their activities could be that the different DILPs interact differentially with the single Drosophila Insulin receptor. In other words, their binding affinities towards the single receptor might differ. Indeed this could be seen in a competitive binding assay between DILP2 and DILP3 (using I^{125}-human insulin as reference). DILP 2 was able to displace radioactive human Insulin (from the surface of the Kc cells) at a lower concentration as compared to DILP 3 (Fig. 9d). This sensitive assay has been widely used to compare and determine the affinities of Insulin like molecules towards the receptor. Hence, the differential potentials of the DILPs to activate the receptor could be due to their differential binding affinities towards the receptor.

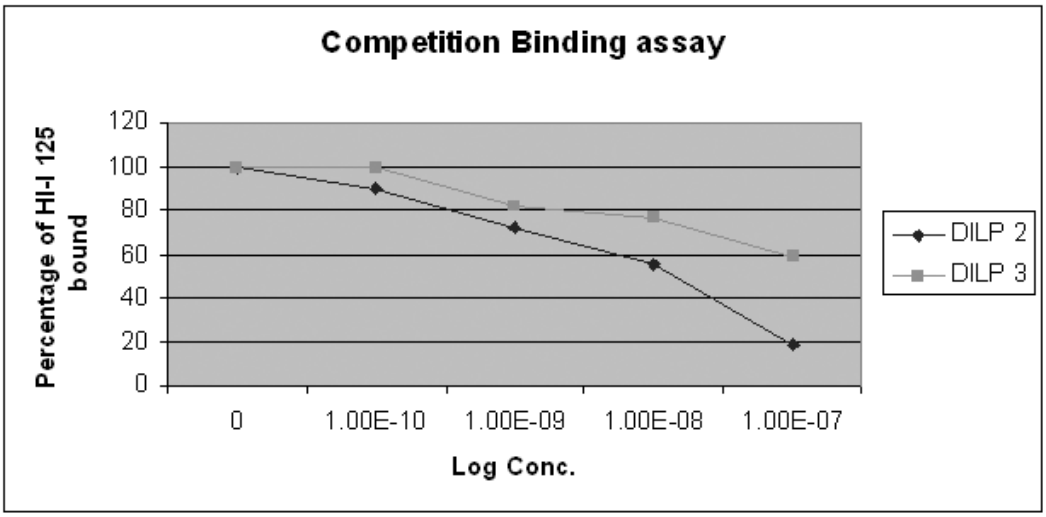


Figure 9d shows non-linear binding affinity curves of DILP 2 and DILP 3 to the Drosophila insulin receptor in Kc cells.

To test the activity of the DILPs on Drosophila tissues directly, transgenic flies expressing tGPH reporter were used [50]. The pleckstrin homology (PH) domain of the Drosophila homolog of general receptor for phosphoinositides-1 (GRP1) was fused to green fluorescent protein (GFP), generating a protein called GPH (GFP-PH domain) PH domains from mammalian GRP1 genes bind specifically to phosphatidylinositol-3,4,5-P3 (PIP3). Since PIP3 generally resides in lipid membranes, particularly the plasma membrane, GRP1 is recruited to membranes when PI3- kinase activity raises cellular levels of PIP3. Fusion proteins containing the GRP1-PH domain are likewise recruited to plasma membranes by binding PIP3, and thus serve as *in situ* reporters for PI3K activity. To check if DILP activity can raise the level of PI3K activity *in vivo*, Drosophila fat body was stimulated with each of the purified DILPs and checked for reporter localisation (Fig 9e).

Figure 9e shows localisation of tGPH reporter in fat body of starved larvae (A) or starved larval fat body when stimulated with DILP 2 (B), DILP 3 (C), DILP 4 (D) or DILP 5 (E). Stimulation with DILPs shows a clear membrane localisation of the tGPH reporter to the membrane as compared to the non-stimulated control (A). Hence, the synthesized peptides are capable of activating the insulin pathway *in vivo.*

Hence, from the above observations one can conclude that atleast four of the seven Drosophila Insulin-like peptides are capable of activating the insulin signaling cascade in cell culture and *in vivo*. The DILPs show variable potencies in activating the receptor which could be explained by their differential binding affinites towards the receptor.

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CHAPTER 3

Effect of DILPs on body size, cell size and cell division

Introduction

The term growth has been widely used in biology to describe processes including progression through a developmental program, cell differentiation, cell division and increase in mass. Here, the term growth is used solely to indicate mass increase of individual cells or tissues or whole organism. The control of entry into and passage through the cell division cycle has been analysed extensively and is now understood in some depth [1]. Less attention has been paid to the regulation of growth.

The growth of an organism throughout its life and attainment of a particular size is a very complicated mechanism to understand. Research has shown that growth factor signaling pathways play an important role in this mechanism. The signaling pathways activated by insulin and insulin-like growth factors (IGFs) in vertebrates have been implicated in the regulation of various processes, including cell growth and proliferation [2, 3]. Certain components of these pathways are conserved in invertebrates and have recently been shown to modify growth and cell size in Drosophila [4, 5].

Role of IGF signaling in growth and cell cycle progression in the mammalian system

Recent advances in understanding cell-cycle control mechanisms have been applied to outline the molecular mechanism through which IGFs regulate cell cycle progression. As described in detail in the introduction (page-), binding of IGF 1 or IGF 2 to the cognate IGF 1R triggers a series of events ultimately leading to the activation of important kinases like PI3K/Akt and MAPK. Surprisingly, the specific pathways activated by IGF 1 and the roles of these pathways in mediating the proliferative response to IGF 1 depend on the cell type. For example, IGF 1 induced cellular proliferation in myoblasts and adipocytes is mediated by the Ras-Raf-MAP kinase pathways [7, 8]. In human intestinal smooth muscle cells [9, 10], IGF 1 elicits growth by activation of both PI3K and the MAPK ERK1/2 pathways, whereas in MCF-7 (a cell line derived from a human breast tumor), the PI3K path way rather than the MAPK-ERK1/2 pathway seems to be an essential step in proliferative signaling of IGF 1 [11]. Also, IGF-I stimulation in human neuroblastoma cells leads to cell cycle progression from G1 to S through both activation of MAPK ERK1/2 [12] and PI3K signaling pathways [13].

Four active phases of the cell cycle are recognized: G1 phase, a pre-DNA synthesis phase in which cells become committed to enter S-phase; S phase, wherein DNA synthesis and chromosomal replication occur; G2 phase, the premitotic gap; and M phase, mitosis. Cells that are not cycling (and not suspended in a specific cell cycle phase) are in the G0 state. The entry of cells from G0 to the G1 phase and from G1 to S phase are the events most tightly regulated by growth factors such as the IGFs.

IGF Axis and the G0/G1 Phase

Ribosome biosynthesis is a key process in cell proliferation. Before entering the cycle, cells need to accumulate sufficient translational machinery, mainly ribosomes, to ensure the rapid translational rates through the cell cycle. This is accomplished at least in part by the phosphorylation of S6 kinase [14]. Once the pool of ribosomes has been achieved, the system is desensitized, either by negative regulators of S6 kinase or by the size of the ribosomal pool. S6 kinase is a serine/threonine kinase activated by growth factor including IGF 1[15], and plays a central role in cell growth and proliferation by mediating the phosphorylation of the 40S ribosomal protein, S6, thereby enabling efficient translation of 5'- terminal oligopyrimidine tract mRNA (5'-TOPs). This class of mRNAs encodes numerous components of the protein synthesis machinery – ribosomal proteins and elongation factors. Thus, IGF 1 by activating S6 kinase may facilitate the transition G0 to G1 phase and also the G1 phase. Indeed, for cells that are actively cycling, ribosomal biogenesis occurs in G1 phase.

Description of the G1/S phase

In normal cells, the transition from G1 to S phase requires the activity of two classes of CDKs, CDK4/6 and CDK2. CDK4 and CDK6 are thought to be involved in early G1, whereas CDK2 is required to complete G1 and initiate S phase. The CDKs are a group of serine/threonine kinase that form active heterodimeric complexes after binding to cyclins, their regulatory subunits [16, 17]. As cells emerge from quiescence in response to mitogenic stimuli such as IGFs, D-type cyclins (cyclins D1, D2 and D3) are synthesized and associate with CDK4/6. This cyclin D-CDK4/6 complex hyper-phosphorylates the retinoblastoma (RB), leading to the release of the transcription factor E2F [18-20]. The free transcription factor E2F then participates in the generation of proteins required for the G1/S transition, such as cyclin E [21]. Cyclin E binds then to CDK2 contributing to kinase activation and G1 to S phase progression. CDKs activity is regulated by various mechanisms that include

phosphorylation [16, 17] and association with both positive (mainly Cyclins) and negative (known as CDKIs, cyclin dependent kinase inhibitors) regulatory proteins.

IGF system and cyclin/CDKs

In rat L6E9 myoblasts, IGF 1 increases cyclin D1 and CDK4 gene expression and consequently the Rb phosphorylation [24]. Also, IGF 1 induces cyclin D1 expression in MCF-7 through the PI3K/ Akt pathway, not the MAPK pathway [25, 26]. Interestingly, this effect of IGF 1 on cyclin D1 expression in MCF-7 is observed by increasing the cyclin D1 mRNA stability [27]. Besides stimulating cyclin D1 transcription, the PI3K/Akt signaling pathway can also increase cyclin D1 levels via enhanced translation of cyclin D1 transcripts [28] and modulation of cyclin D1 protein turnover by inhibiting GSK-3-mediated cyclin D1 phosphorylation [29]. Moreover, in MCF-7S, IGF 1 triggers nuclear accumulation of cyclin D1 [30]. Thus, in breast cancer cell lines, the IGF1-induced PI3K/ Akt pathway can regulate cyclin D1 expression at multiple levels – IGF 1 can induce both elevation of cyclin D1 protein and mRNA, and nuclear accumulation of cyclin D1 during the G1 phase. The influence of the PI3K/Akt pathway on cyclin D expression is more evident when considering the role of PTEN, a lipid phosphatase known to counteract PI3K/ Akt signaling. PTEN inhibits cell cycle entry by blocking G1 to S phase progression through at least downregulating cyclin D. For example, in endometrial carcinoma cells that lack endogenous wild-type PTEN, the introduction of PTEN gene induces cell cycle arrest by specifically decreasing the cyclin D3 levels [31]. Also, expression of PTEN is associated with decreased expression of cyclins D3 and A and consequently a hypo- phosphorylation of Rb in glioma cell lines [32]. Thus, PTEN inhibits the IGF 1 effects on cyclin expression by blocking the PI3K/Akt pathway. However, the mechanisms involved in the induction of cyclin D by Akt in response to IGF 1 are still unknown. Downstream targets of Akt are likely involved. Recently, a study using microarray analysis has shown that cyclin D1 and cyclin D2 were transcriptional targets of FOXO1 (FKHR), one of the main substrates of Akt [33]. Taken together, the PI3K/Akt is a crucial signaling pathway involved in the expression of cyclins.

The MAPK pathway, the other main pathway downstream of IGF-1R, also plays an essential role in the regulation of cyclin D expression and cell cycle progression [34-36]. Consistent with these observations, PTEN, by inhibiting MAPK phosphorylation, inhibits cyclin D1 expression and cell cycle progression in response to growth-factor stimulation in MCF-7 [37].

Role of Insulin pathway in growth and proliferation in Drosophila

Drosophila imaginal discs are simple epithelial structures in which growth is accompanied by cell division. They grow and are patterned during larval stages, and then differentiate during the pupal period, giving rise to most of the adult epidermis [38]. Imaginal discs grow to highly reproducible sizes, and classical experiments have shown that their growth is influenced by factors that are extrinsic of and intrinsic to the disc [39, 40] Experiments on discs in Drosophila have shown that the division machinery can be activated independently of the growth machinery [41]. In the past couple of years several groups working on Drosophila have shown that the mutation of components on the insulin/PI 3-kinase signalling pathway in Drosophila alters cell size and organism size [4-6]. Some of the functions ascribed to this pathway in mammals are consistent with its ability to regulate growth in Drosophila, as discussed above. Null mutations in most of the components of the pathway are lethal. However, hypomorphic mutations in the Drosophila insulin/IGF receptor gene, *dInr* [42], produce small flies that have small cells. Similar effects on organ size and cell size are induced by the expression of transgenes that modulate the activity of dPKB or the Drosophila Class IA PI 3-kinase, Dp110, during eye and wing development [43-45]. More recently, it has been shown that this pathway is antagonized by dPTEN [46-48]. Hence, this is a signaling pathway that, at least at the gross phenotypic level, affects net growth and alters cell size. Flow cytometry analysis of mutant clones or gain-of-function clones as compared to the wild type twin spots, a technique developed by Neufeld and colleagues [41], has definitively shown that modulating signalling through the insulin/PI 3-kinase pathway does alter the size of dividing cells. For example, removal of CHICO or DS6K, inhibition of the activity of dAKT1 or Dp110, or overexpression of DPTEN, reduces cell size. In contrast, removal of dPTEN or overexpression of Dp110 or dAKT1 increases cell size [43, 44, 48-50].

Another important issue is whether the insulin/PI 3-kinase signaling pathway alters cell number and hence influences the amount of cell division that occurs during imaginal disc development. Wings from flies that completely lack *chico* possess fewer cells than wild-type wings do [50]. Similarly, overexpression of Dp110 in a large area and during an extended period of wing development can result in a mild increase in cell number [45]. From these observations, it is unclear whether this pathway directly alters the activity of cell cycle regulators to influence cell division. The effect on cell number could be indirect and merely reflect the fact that, ultimately, inadequate biosynthesis will not allow the normal number of cell divisions to occur whereas increased biosynthesis might indirectly allow extra cell divisions.

Other experiments have shown that clones of imaginal disc cells overexpressing Dp110 or dAkt1 are larger in area than control clones but contain the same number of cells [43, 44]. This observation indicates that increasing signalling via the insulin/PI 3-kinase pathway can promote growth without having a proportional impact on cell division. Edgar and co-workers have obtained similar results by overexpressing dMyc [51] or activated dRas1 [52], although whether these molecules promote growth by interacting with the insulin/PI 3-kinase signaling pathway has not yet been addressed. In addition, flies without dS6 kinase have the same number of wing cells as wild-type flies do, which suggests that dS6 kinase modulates imaginal disc growth and cell size without directly influencing the number of cell divisions that occur during development [49].

The above observations appear in keeping with the model that a change in the ratio of signals carried to the growth machinery and cell division machinery alters cell size. The insulin/PI 3-kinase signalling pathway would carry the signal to the growth machinery. However, this model does not explain the subtle effects of this pathway on cell number. Perhaps a minor component of the signal is diverted towards the cell division machinery. Alternatively, the growth signal synergize with other signals that drive cell division.

Data from other experimental systems suggest that PI 3- kinase can increase the activity of cell cycle regulators, but that PI 3-kinase activation alone is not sufficient to promote division [53, 54]. For example, in rat 3Y1 cells, expression of activated PI 3-kinase under low-serum conditions activated E2F, but the cells arrested in mid-S-phase and died by apoptosis. These effects were rescued by higher concentrations of serum, which lead to uncontrolled proliferation and growth, presumably because PI 3-kinase-induced E2F activation synergizes with other signaling pathways to promote 3Y1 cell division [53]. Similar factors might come into play when Dp110 is over expressed in Drosophila imaginal discs. This artificial situation will not precisely mimic the context in which Dp110 is usually activated. During normal development, the insulin/PI 3-kinase signaling pathway is likely to synergize with other signaling pathways, which are activated in parallel, to influence cell division.

In contrast to the inability of the insulin/PI 3-kinase signaling pathway to influence cell division directly, several observations demonstrate that insulin/PI 3-kinase signaling affects the rate at which cells progress through the cell cycle in Drosophila. *dS6 kinase* and *chico* mutant larvae are developmentally delayed and grow more slowly, and yet the resulting flies have normal or reduced numbers of cells [49, 50]. This indicates that the cell cycle is lengthened in these organisms. In addition, the analysis of cell number in clones, a short

period after their induction, indicates that cells with reduced dS6 kinase or Dp110 activity divide more slowly [49, 50].

The analysis of cell cycle profiles by flow cytometry has suggested that altering signaling via the insulin/PI 3-kinase signaling pathway disproportionately influences progression through different phases of the cell cycle. Overexpression of Dp110 or loss of dPTEN reduces the proportion of cells in G1 and increases the proportion of cells in S phase and at G2-M, without affecting cell doubling time [44, 48]. These observations suggest that increased signaling via Dp110 is sufficient to hasten entry into S phase. Although similar results have been obtained by modulating dRas1 and dMyc activity [51, 52], effects on cell cycle phasing have not been observed when the activity of other insulin pathway components is altered. The slowing down of the cell cycle in *chico* and *dS6k* mutant cells is accompanied by a proportional extension of all phases of the cell cycle [50, 49]. Thus, the observation that Dp110 overexpression or loss of PTEN can hasten S phase entry might reflect the fact that these interventions somehow have a stronger impact on the signaling pathway than the other experimental interventions examined do. Alternatively, the pathway might branch and be less linear. Note that the effect on growth and the cell cycle of dInR, which is upstream of Dp110, has not yet been characterized in any detail.

These results are comparable with observations on growing yeast cultures. Budding yeast pass rapidly through G1 when nutrients are abundant, and growth is rapid, but pass slowly through G1 when nutrient conditions are poor [55, 56]. These observations support the idea that entry into S phase is regulated by growth, and that high rates of growth hasten S phase entry. Thus, it would be interesting to investigate whether the same is true in flies and whether the ability of Dp110 and DPTEN to influence G1-S is dependent on their ability to modulate growth. Furthermore, this pathway may be regulated in flies, as it is in other organisms, by nutrition [44, 45, 50]. Since expression of Dp110, dRas1 or dMyc can promote growth and S phase entry but not division, their ability to increase cell size might diminish if entry into M phase could be hastened.

The data discussed so far demonstrate that the insulin/PI 3-kinase pathway in Drosophila promotes growth without proportionally stimulating cell division. However, modulating the activity of this pathway can affect cell cycle progression (i.e. by slowing cell division or altering cell cycle phasing). These effects on cell cycle progression may be best-explained by the ability of this pathway to promote growth.

In order for a tissue to grow, increased biosynthesis must occur, for which increased protein synthesis is likely to be critical. The observation that dividing cells have an increased translational capacity whereas quiescent cells have a reduced translational capacity is consistent with this requirement for increased protein synthesis [57, 58]. Translation initiation is thought to be rate limiting in protein synthesis and is regulated in multiple ways [59], some of which are influenced by insulin signaling [60]. One way in which insulin signaling can increase translation initiation is by activating the eukaryotic translation initiation factor eIF4E [61]. eIF4E binds to the 5' cap structure of all eukaryotic mRNAs and is a subunit of the eukaryotic translation initiation factor eIF4F. eIF4F activity brings the 5' end of mRNAs to ribosomes [62]. In addition, eIF4F possesses RNA helicase activity that can help dissolve mRNA secondary structures, thereby enabling translation. The regulation of eIF4E activity is complex and mediated both by its association with a family of eIF4E-binding proteins (the 4EBPs) and by phosphorylation. One of the effects of insulin/PI 3-kinase signaling is to promote 4E-BP1 phosphorylation [63]. This results in the disassociation of 4EBP- 1 from eIF4E, allowing eIF4E to bind to the other subunits of eIF4F [64]. Insulin can also stimulate translation by activating S6 kinase. In addition, insulin-stimulated PI 3-kinase activation can activate the translation initiation factor eIF2 [61]. Insulin/PI 3-kinase signaling can thus increase rates of translation initiation in vertebrates through several mechanisms. Although this pathway is likely to have similar effects on translation in Drosophila, this has not yet been proven.

On one hand there are hints that PI3K or MAPK kinase signaling in response to IGF receptor activation can directly affect cell cycle machinery in certain mammalian cell lines, as discussed above. Some studies which that PI3K or MAPK pathway alone cannot activate the whole cell cycle machinery and that they synergize with other pathways to achieve this.

In Drosophila, with the presence of seven ligands and one receptor, there was a need to establish and determine growth affecting functions of the DILPs. Brogiolo et al [65] showed that ubiquitous overexpression of DILP 2 using the UAS-Gal4 system increased body weight. This was due to an increase in cell number and cell size. Rulifson et al [66] and Ikeya et al [67] showed that ablation of the DILP expressing neurons during development gives rise to smaller pharate adults with reduced body cell number and size. These studies suggested that the DILPs do play a role in cell growth and cell division. Growth regulating functions of other DILPs were not clearly established.

To answer this I used the Gal4-UAS system to overexpress the *dilp* genes during development. A measure and comparison of the body weight would tell if any of the DILPs has negative impact on growth.

Purification of DILP 2, 3, 4 and 5 made it easier to also compare their effects on growth and cell cycle in cell culture. I used two techniques, FACS analysis and BrdU incorporation, to study the effects of DILPs on cell growth and cell cycle.

FACS analysis was used for an estimation of cell size and analysis of the cell cycle. Fluorescence Activated Cell Sorter has become one of the important tools to visualize and quantify cell cycle progression of cells in culture. The term flow cytometry derives from the measurement of single cells as they flow by a series of detectors. The fundamental concept is that cells flow one at a time through a region of interrogation where multiple biophysical properties of each cell can be measured at rates of over one thousand cells per second. The high through-put of cells allows for rare cells, which may have inherent or inducible differences, to be easily detected and identified from the remainder of the cell population. In order to make the measurement of biological/biochemical properties of interest easier, the cells are stained with fluorescent dyes which bind specifically to cellular constituents. The dyes are excited by the laser beam and emit light at longer wave lengths, this is detected by photomultipliers and these analogue signals are converted to digital so that they may be stored for later display and analysis. Light scattering measurements are widely used for the estimation of cell size as the intensity of light scattered at small angles from an incident beam is proportional to particle volume. The light scattered perpendicularly to the laser incidence is proportional to the cell shape and optical homogeneity, and can be considered as an estimate of cell complexity and granularity [69].

The immunocytochemical detection of bromodeoxyuridine (BrdU) incorporated into DNA is a powerful tool to study the cytokinetics of normal and neoplastic cells. *In vivo* labeling of tumor cells with the thymidine analogue BrdU and the subsequent detection of incorporated BrdU with specific anti-BrdU monoclonal antibodies is an accurate and comprehensive method to quantitate the degree of DNA synthesis. BrdU is incorporated into the newly synthesized DNA of the S-phase cells and can thus provide an estimate for the fraction of cells in S-phase [70].

Two studies assessed the effects of insulin on the growth and division of Drosophila cells. Both looked at the effects of Insulin/PI3K/ERK signaling in response to human or bovine insulin on Kc cell size and cell number. Hilliker et. al. [71] in 2004 reported a 2 fold increase

in BrdU incorporation in Kc cells, which was inhibitable by either PI3K or ERK inhibitors. They showed an increase in cell size, which was however only inhibitable by PI3K inhibitors indicating that ERK activation in Drosophila does not affect cell size, although it affects cell cycle progression. Another study published Kim et al. in 2004 [72] showed that G1 to S phase transition of S2 cells was increased after insulin stimulation and could be blocked by dPI3K inhibitors. They also showed that insulin stimulated size increase was blocked by inhibitors of dPI3K pathway but not dERK, suggesting that size increase in Drosophila cells is mediated by dPI3K but not through dERK.

Both studies concluded that Insulin can stimulate a cell size increase and cell cycle progression in Drosophila culture. The studies also implicate that the effect of insulin on cell cycle progression is clearer in cell culture as compared to *in vivo* data discussed above.

Since purified DILP 2-5 were now available, I decided to study the effects of DILP stimulation on cell growth and cell division using Drosophila cell lines.

Materials and Methods

dilp overexpression

UAS constructs of *dilp1-7* were prepared by inserting PCR- amplified genomic DNA encompassing the coding regions of each of the *dilp* genes into the pUAST vector [67]. Several independent transgenic lines were obtained for each construct. For *Gal4* expression *armadillo-Gal4*, *hs-Gal4* and *Act- Gal4* lines were used. Each of the UAS- *dilp X* line was crossed with the above mentioned *Gal4* lines. In case of *hs-Gal4*, one hour heat shock (at 37°C) was given every 12 hours during entire course of development. In each case freshly eclosed males and females of right genotype were collected separately and allowed to mature for three days. Weights of male and female individuals were measured and analyzed.

BrdU incorporation

Kc cells were grown to 80% confluence and were collected and washed with serum free Schneider's medium. They were suspended in serum free medium and plated in six-well plates containing cover slips. After 24 hours, medium was decanted and the cells were provided fresh serum free medium, serum-free medium plus DILP 2, 3, 4 and 5 or normal medium. The cells were incubated for another 12 hours in six well plates containing cover

slips. In the last 5 hours, BrdU was added to a final concentration of 1ug/ul. At the end of the incubation, the medium was gently removed and cells were washed twice with ice cold PBS. Cells were fixed with 100% (ice cold) Methanol, washed twice with PBS and then blocked with blocking solution, PBT (0.1%BSA, 0.1% Triton in PBS) for 1 hour. Mouse anti-BrdU antibody (Amersham, Catalog no. RPN202) was added at a dilution of 1:1000 in blocking solution for 1 hour. After washing with PBS thrice, FITC labeled secondary anti-mouse antibody (Jackson Immunoresearch, Catalog no. 715095150) was added at a dilution of 1:1000 in blocking solution for 1 hour. Cells were washed thrice with PBT and were stained with DAPI for 1 hour. The cover slips were mounted using Vecta Shield (mounting medium, H1000). Total no. of cells (DAPI stained) and BrdU incorporated cells (detected by FITC fluorescence) were counted on 10 different areas on the slide at 10X magnification. Numbers of BrdU incorporated cells were plotted as percentage of total number of cells.

Cell cycle and cell size analysis

Kc cells were grown in full Schneider's medium for two days before they were splitted again (1:3) in fresh normal medium. After 24 hours, 2 million cells were seeded into six-well plates in fresh medium. The cells were then stimulated with either DILP 2 or 3 and collected after 0hr, 4hrs, 8hrs and 10hrs of stimulation. For fixation and propidium iodide staining, the cells were collected in falcon tubes and washed twice with PBS and fixed with 70% ethanol at 4°C overnight. Ethanol was discarded and fixed cells were washed twice with PBS and finally resuspended with 500µl blocking solution (0.1% BSA in PBS). 1µg/µl RNase and 10 x propidium iodide solutions were added and the falcon tubes were incubated at 37° C for 30 minutes before analyzing in Fluorescence activated cell sorter (Beckman and Coulter). Three samples were collected for the control and DILP-stimulated cells at each time point. Forward and side scatter of ungated and gated cells were determined for cell cycle and cell size analysis. Percentage of cells in various phases of the cell cycle was calculated and plotted.

d*ilp*-RNAi experiment

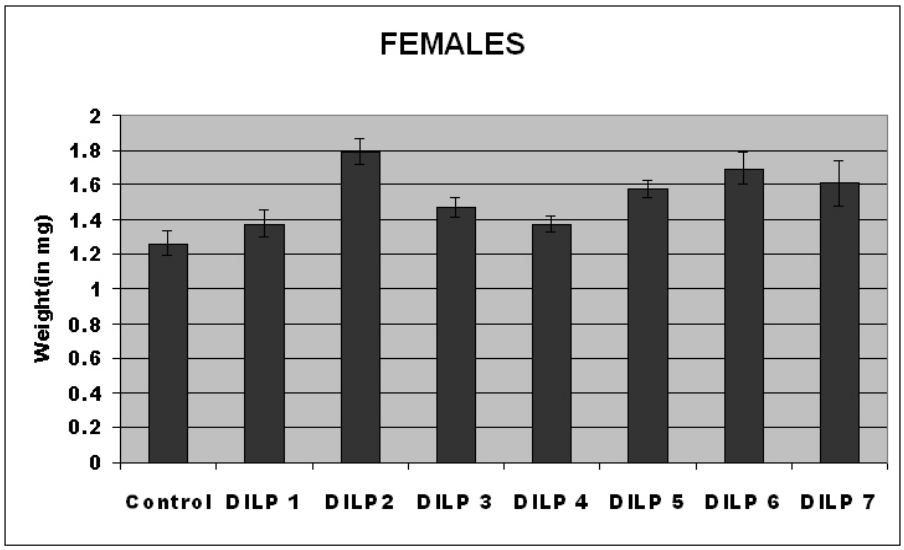
UAS-*dilp2*RNAi and UAS-*dilp3*RNAi transgenic lines were constructed as published by Kalidas et. al. [76]. Briefly, full length genomic region of *dilp 2* (containing one intron and two exons), was cloned in front of inverted *dilp2* cDNA. This construct was then cloned into a pUAST vector resulting in pUAST-dilp2RNAi vector. Transgenic flies were generated by injecting this vector. For overexpression in mNSCs, UAS-*dilp2*RNAi lines were crossed with *dilp2*-GAL4 flies. Growth effects were checked by weighing 3 day old adult males. UAS-*dilp3*RNAi lines were constructed and tested similarly.

Results and Discussion

Overexpression of DILP 1-7 promotes growth

Characterizing and quantifying the effects of DILPs on growth in Drosophila was the most straight forward experiment to establish similarities or differences between various DILPs. It is possible, for example, that some of the DILPs act as receptor antagonists, as has been postulated for some insulin-like peptides in C. elegans. [74]. DILP 2 was already shown to be a growth promoter [65]. I tested whether DILP 1 and DILP 3-7, like DILP2, also promote growth when expressed ubiquitously throughout development. As shown in Fig. 1a and 1b, expression of dilp genes under the control of the weak, ubiquitous *armadillo-Gal4* (*arm-Gal4*) driver caused a statistically significant increase in body size of both males and females. The largest increase was observed by *dilp2* overexpression resulting in a 51% weight gain in males (Fig 1a). This increase in size is the result of increase in cell size and cell number [65]. Constitutive high levels of expression of the dilp genes using an *actin-Gal4* driver caused lethality in the case of *dilp 2* and slightly higher weight increases with other dilp constructs (data not shown). Interestingly, expression of human insulin gene either under heat shock or actin control did not cause a significant increase in body size or lethality (data not shown), despite its known growth-promoting effect in tissue culture [65]. It is possible that human preproinsulin is not processed correctly in Drosophila cells.

Hence it can be concluded that, under the conditions tested, all Drosophila insulin genes can promote growth in the organism. The extent of growth promotion, as measured by body weight, is varying. Either the transgenes were overexpressed with different efficiencies or the ligands differ in their efficiency of receptor stimulation. Levels of endogenous or overexpressed DILPs could not be detected through Western blotting. The rabbit polyclonal antibodies which are able to detect endogenous DILP 2 and DILP 7 in specific neurons in the Drosophila larval brain (Chapter 2) but not in Western blotting probably due to low molecular weight low expression levels of the DILPs.



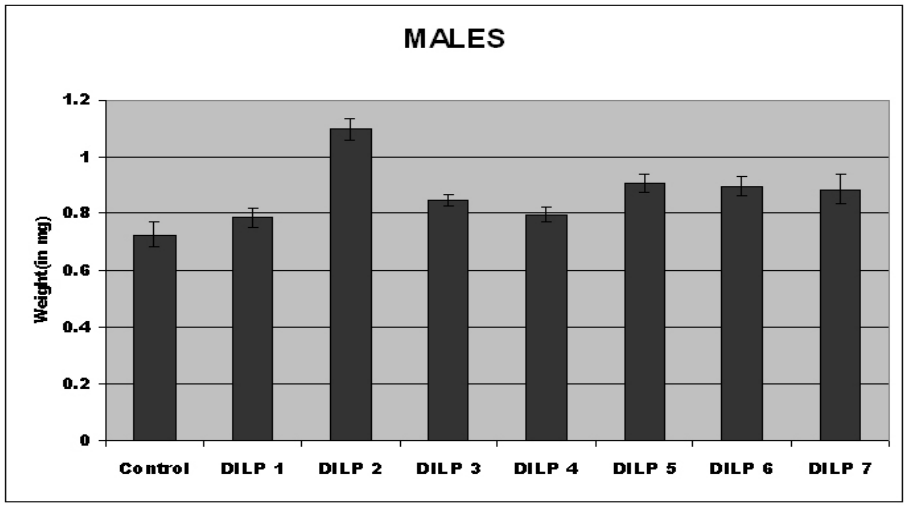


Figure 1a and 1b show body weights of adult Drosophila males and females overexpressing *dilp 1-7* with *armadillo-Gal4*.

dilp RNAi experiments

Up to date no loss-of-function mutants have been reported for any of the *dilp* genes. Ablation of neurons, which express some of the *dilps,* are the only experiments which suggest possible functions of the DILPs in Drosophila. To create loss-of-function like conditions for *dilps* in Drosophila, I constructed UAS-*dilp*RNAi transgenic flies which can theoretically form hairpin transcripts for the *dilps* and thus lead to their degradation. These constructs, which contain genomic region (containing introns) fused to inverted cDNA of the gene, were shown to work well in Drosophila neurons [76]. However, the UAS-*dilp2*RNAi or UAS-*dilp3*RNAi when overexpressed in *dilp* expressing neurons did not show any apparent effect on adult male weight (Fig. 1c). Immunostaining for DILP 2 in mNSCs in *dilp2*GAL4-UAS *dilp2*RNAi

did not show any obvious reduction in the levels of DILP 2 as compared to *dilp2*-GAL4 animals (data not shown).

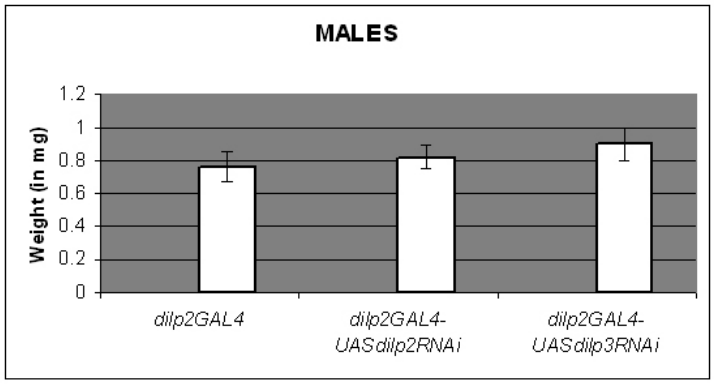


Figure 1c shows adult male weight (in mg) in control (*dilp2*-GAL4), *dilp2*GAL4-UAS *dilp2*RNAi or *dilp2*GAL4-UAS *dilp3*RNAi. UAS-*dilp* RNAi lines do not show any apparent decrease in body weight of males as compared to the control.

DILP 2-5 promote G1 to S phase progression

The two studies on Drosophila cells (as discussed in the Introduction) [71, 72] clearly demonstrate positive growth and proliferation effects of bovine or human insulin in Drosophila cell culture. To study if DILP 2-5 stimulation can promote cell cycle progression from, I performed BrdU incorporation and flow cytometry analysis by using Kc cells. The aim was also to see if DILPs might differ in their effects on G 1 to S phase transition.

When Kc cells were starved in serum-free medium for 12 hours, and then were stimulated by DILP 2, 3, 4 or 5, they showed an increase in the number of cells which incorporated BrdU as compared to the unstimulated cells (Fig 2a-b). The concentration of each DILP used here leads to maximum activation of downstream kinases S6 kinase and dPKB (Chapter 1) but still there is a differential effect on cell proliferation. Figure 2a-b show that DILP 2 has stronger effect on G1 to S phase transitioning of Kc cells, followed by DILP 3 and then with DILP 4 and DILP 5. One possible explanation for the differential effects of DILPs on G1-S phase progression could be that DILP 2 probably is capable of activating other cell cycle progression pathways in Drosophila cells either via Insulin receptor or other receptors, which the other DILPs are not capable of doing.

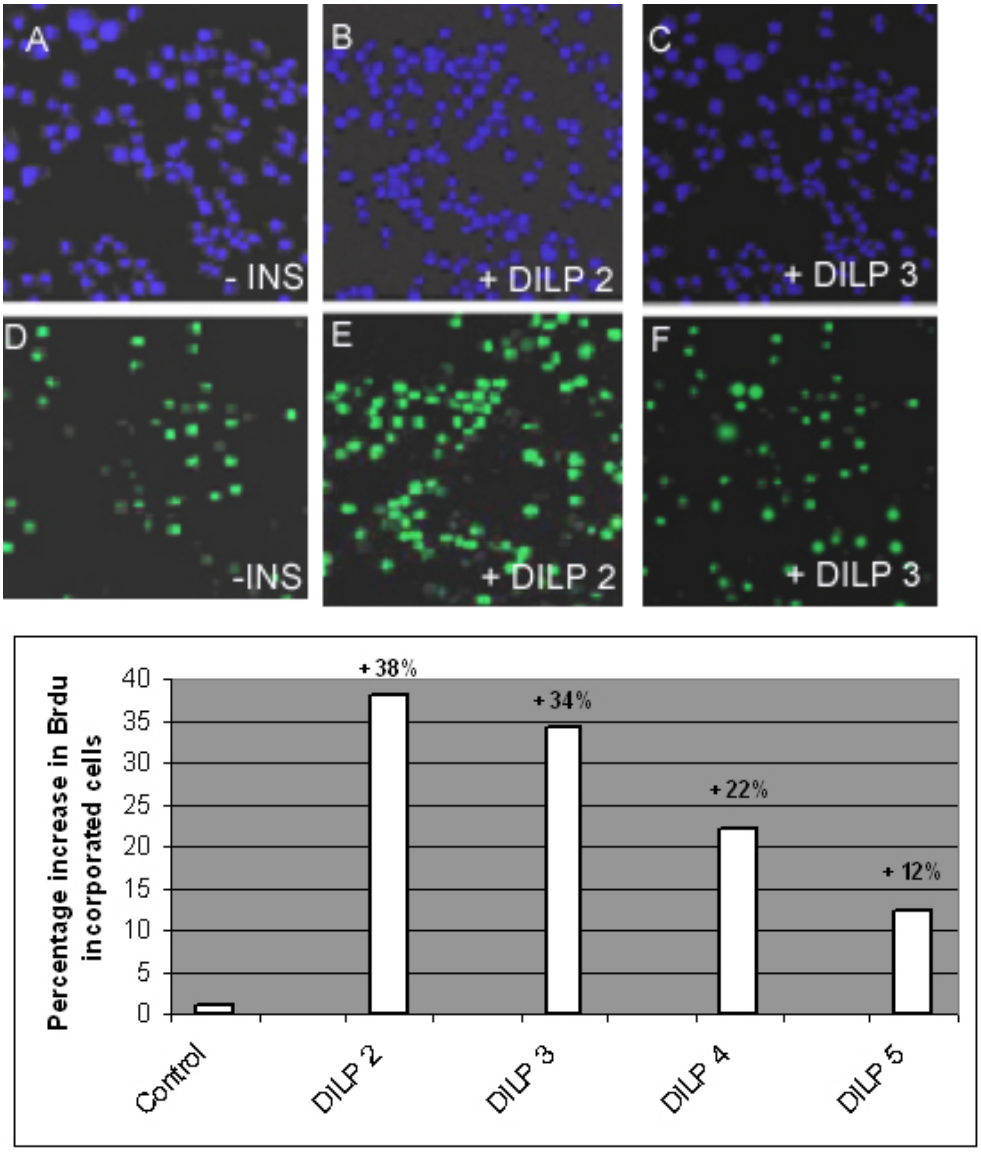


Figure 2a shows percentage increase in BrdU incorporated Kc cells when stimulated with DILP 2-5 as compared to non stimulated cells.

Cell cycle analysis by flow cytometry also revealed similar effect of DILP 2 and DILP 3 stimulation in transitioning of cells from G1 to S phase. Figure 3a and 3b show increase in the percentage of Kc cells in S phase upon stimulation with DILP 2 and DILP 3. There is approximately 36% increase in cells in S-phase and approximately 38% increase in cells stimulated with DILP 3. Hence, it can be concluded that both DILPs can promote entry of Drosophila cells in S-phase.

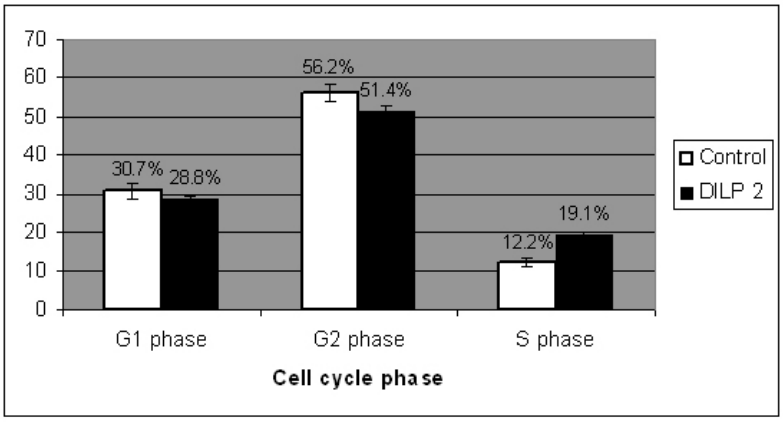


Figure 2b shows percentage of Kc cells in various phases of the cell cycle (G1, G2 or S phase) as shown by flow cytometry. The graph compares DILP 2 stimulated cells with non stimulated controls.

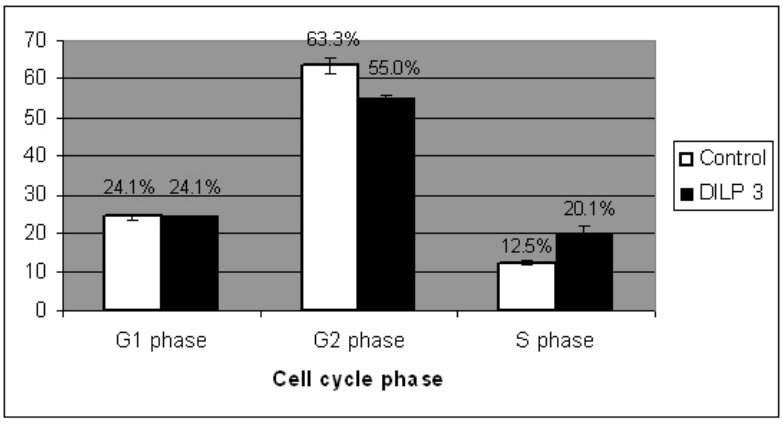


Figure 2c shows percentage of Kc cells in various phases of the cell cycle (G1, G2 or S phase) as shown by flow cytometry. The graph compares DILP 3 stimulated cells with non-stimulated controls.

DILP 2 and 3 promote increase in cell size

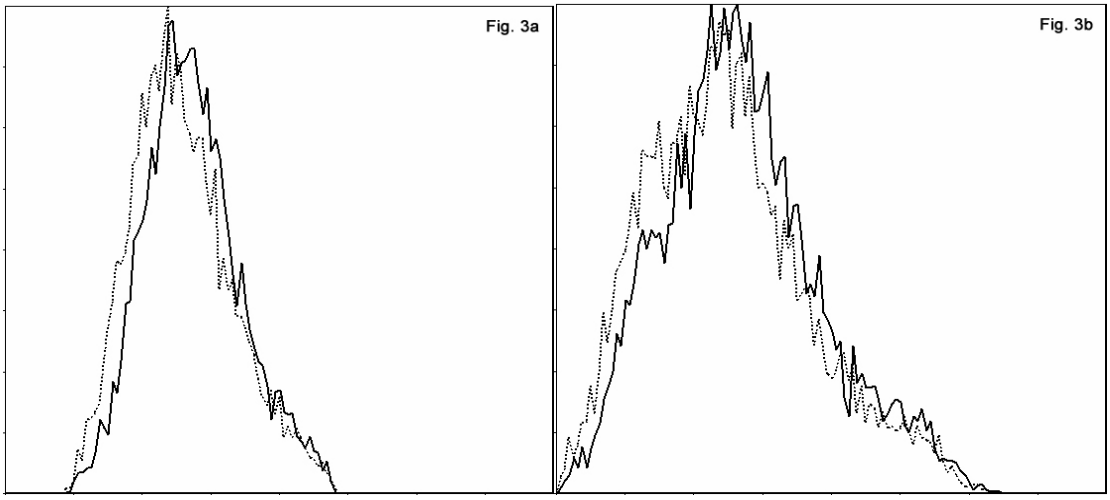


Figure 4a and 4b compare the forward scatter of unstimulated Kc cells (punctated boundary) to Kc cells stimulated with DILP 2 or DILP 3 (normal boundary). The forward scatter of DILP stimulated cells is slightly shifted towards the right indicating that DILP stimulated cells are larger in size as compared to non-stimulated cells.

Taken together, the results suggest that all DILPs promote growth *in vivo*. Stimulation by some of the DILPs can increase cell size and encourages of cell division as seen in cell culture assays. None of the DILPs in these experiments showed a negative effect on growth suggesting that none of them acts as antagonist.

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CHAPTER 4

Effect of DILPs on Glucose uptake

As discussed in the Introduction, one of the primary metabolic responses mediated by Insulin is the stimulation of facilitated glucose transport in muscle and adipose tissues. Insulin in mammalian cells is known to stimulate and translocate Glucose transporters to the membrane where they facilitate the transport of glucose into the cells.

Glucose transporters (GLUT proteins)

Most mammalian cells depend on a continuous supply of glucose not only as a precursor of glycoproteins, triglycerides and glycogen but also as an important source of energy by generating ATP through glycolysis. Glucose is a hydrophilic compound; it cannot pass through the lipid bilayer by simple diffusion, and therefore requires specific carrier proteins to mediate its specific transport into the cytosol. There is an energy-dependent Na^+/glucose co-transporter in the polarized epithelial cells in the lumen of small intestine and in the proximal tubules of the kidney. Exclusively in the aforementioned cells this protein uses the movement of Na^+ down its electro-chemical gradient to drive the uptake of glucose.

A ubiquitous glucose transport system also exists. All mammalian cells contain one or more members of the facilitative glucose transporter gene family named GLUT (Table 1). These transporters have a high degree of stereo selectivity, providing for the bidirectional transport of substrate, with passive diffusion down its concentration gradient. GLUTs function to regulate the movement of glucose between the extracellular and intracellular compartments maintaining a constant supply of glucose available for metabolism.

Localization and structure of GLUT proteins

The GLUTs are intrinsic membrane proteins which differ in tissue-specific expression and response to metabolic and hormonal regulation [1, 2, 3]. Many different isoforms of GLUTs have been identified (Table 1); all appear to share a common transmembrane topology, having a large (50% of protein mass), highly conserved (97%), transmembrane domain, with a less conserved, grossly asymmetric, non-membrane, cytoplasmic and exoplasmic domains [4]. The transmembrane domain is composed of twelve membrane-spanning-helices, containing a water-filled pathway through which the substrate moves [5, 6]. The cytoplasmic domain

contains a short N-terminal segment, a large cytosolic loop and a large C-terminal segment. The exoplasmic domain contains a large loop bearing a single N-linked oligosaccharide moiety.

Protein	Function	Expression
GLUT 1	Basal uptake	All tissues (abundant in brain and erythrocytes)
GLUT 2	Glucose sensing	Liver, pancreatic islet cells, retina
GLUT 3	Supplements GLUT1 in tissues with high energy demand	Brain
GLUT 4	Insulin responsive	Muscle, fat, heart
GLUT 5	Fructose transport	Intestine, testis, kidney, erythrocytes
GLUT 6	N.D.	Spleen, leukocytes, brain
GLUT 7	Fructose transport	Liver
GLUT 8	N.D.	Testis, brain
GLUT 9	Fructose transport	Liver, kidney
GLUT 10	N.D.	Liver, pancreas
GLUT 11	Fructose transport	Heart, muscle
GLUT 12	N.D.	Heart, prostate

Table 1 (adapted from [135]) summarizes the tissue specific expression and functions of various GLUT proteins.

Physiological functions of GLUT proteins

The physiological functions of GLUT transporters depend on their kinetic and substrate specificities. Several studies have examined the kinetic properties of the isoforms. Results of

transport assays, under equilibrium exchange conditions, show an apparent Km for 3-O-methylglucose transport by GLUT1 of 16.9-26.2 mM [7, 8]. Under the same conditions GLUT4 has a Km of 1.8-4.8 mM [9, 8] and GLUT3 has a Km of 10.6 mM [7]. This means that GLUT3 and GLUT4 have a higher affinity for glucose than GLUT1, ensuring that glucose transport will be maximal in tissues containing these isoforms even when glucose concentrations are low. This is particularly important for the brain, which expresses GLUT3, and relies on glucose as its only source of energy.

GLUT2 has a very low affinity for glucose with a Km for 3-O-methylglucose of 40 mM [7]. Since normal circulating glucose concentration is 3.9-5.6 mM, the rate of transport will be directly proportional to glucose concentration. Therefore, in the postprandial state, when circulating glucose levels are high, there is a net flux of glucose into hepatocytes and pancreatic ß-cells. In contrast, when circulating glucose levels are low, intracellular glucose concentration will increase as a result of glycogenolysis and gluconeogenesis. When the intracellular glucose concentration exceeds the plasma concentration GLUT transports glucose from the liver into the circulation. GLUT2 also functions as a low-affinity fructose transporter, which is consistent with the liver being the primary site for fructose metabolism [7]. GLUT2 is further involved in the anterior transport of glucose supplied by choroidal circulation from the early stages of retinal development [10].

The localization, expression and regulation of the GLUT family proteins are tissue and often cell specific. New GLUT isoforms are continually being discovered and characterized in various cell types. Their involvement in disease states is also continually under review. In cancer cells, which have broken free from the normally tight global regulation, aberrant expression of the GLUT family members provides the energy source required for further uncontrolled proliferation and metastasis. As every cell contains the genes for each GLUT family member we observe in cancer cells the expression of certain GLUT isoforms which, under normal conditions, would never have been expressed in these tissues.

Adipose and muscle tissue

One of the most important, and well established, models of GLUT regulation is the stimulation of GLUT expression and translocation in adipose and muscle tissue by insulin [11; 12, 13]. It is this process that provides the regulation of whole body glucose homeostasis and, when dysfunctional, plays a vital role in diabetes mellitus. GLUT4 is almost completely responsible for insulin-stimulated glucose transport. In rat adipocytes, the most studied cell

system for insulin action on glucose transport, more than 95% of GLUT4 and 30-40% of GLUT1 is associated with intracellular membranes, and is thus nonfunctional. These GLUTs are translocated to the plasma membrane in response to insulin, where they are able to facilitate the transport of substrate [14]. GLUT4 is constantly recycled between the plasma membrane and intracellular storage pool with two discrete first order rate constants, one for internalization (kin) and one for externalization (kex). Insulin causes transporter translocation by reducing kin and increasing kex approximately 3-fold each [15]. Impaired GLUT activity is in part responsible for insulin resistance in human diabetes and obesity [16].

Heart

The rate of glucose utilization in the rat heart is greater than in many tissues such as skeletal muscle, adipose and lung [17]. Cardiac muscle glucose transport and utilization is vital for normal function, a fact illustrated in GLUT4 cardiac knockout mice which show cardiac hypertrophy and other major morphologic heart changes [18]. Moreover, a high rate of cardiac glucose metabolism becomes crucial during ischemia when oxidative phosphorylation is limited. Under basal conditions glucose transport is the rate limiting step in glucose metabolism, however, the element of control shifts to phosphorylation by hexokinase in the presence of insulin [19].

There are two main glucose transporters present in cardiac tissue. Under un-stressed conditions approximately 60-70% of GLUT1 and 10-20% of GLUT4 is localized in the plasma membrane [20]. In cardiomyocytes, GLUT4 and GLUT1 account for approximately 60% and 40% respectively, of total glucose carriers [21]. A number of different stimuli, such as ischemia, insulin and lactate, have been shown to cause translocation of GLUT1 and GLUT4 to the plasma membrane [22-26]. These effects may be crucial in the overall metabolism of glucose since, as mentioned above, under many conditions; transmembrane transport is the limiting step in glucose breakdown in the heart [27-29]. In addition to increased translocation of GLUT4 in response to acute myocardial ischemia, chronic ischemia increases GLUT1 protein content by enhancing GLUT1 mRNA expression [30].

Fasting and diabetes cause a repression of cardiac GLUT1 and GLUT4 protein levels in the rat heart [31] and cardiac sarcolemmal vesicles from diabetic rats show decreased glucose transport [32]. These results suggest a decrease in glucose transporter number at the cell surface and indicate that both fasting and diabetes alter the expression and distribution of glucose transporters. Therefore, it is possible that GLUT depletion and diminished glucose

transport across the cell surface of cardiomyocytes in diabetes could limit glucose availability and lead to myocardial dysfunction.

Brain

Many tissues types can utilize a variety of substrates, such as glucose, lactate and fatty acids, as an energy source. In contrast, the adult mammalian central nervous system relies on glucose as its sole source for ATP production. In order for glucose to reach neurons within the brain it must first cross the endothelium of the blood brain barrier into the interstitial space. From this compartment glucose must be transported across the neuronal plasma membrane using the ubiquitous GLUT1 and GLUT3 isoforms. Brain GLUT1 is a multiple-molecular-weight species ranging between 45-55 KDa [33]. The larger-molecular-weight species are present in microvessels [34], the smaller species are present in vessel-free preparation on brain membranes [35] and an intermediate species is present in the choroid plexus [36]. The differences in molecular weight are due to differences in N-linked glycosylation. The functional effect of the different glycosylation states is not clear although there is evidence suggesting that they are involved in GLUT1 trafficking [37] and substrate affinity [38]. GLUT3 is highly expressed in the brain [39], specifically in neurons [40]. Its relatively low Km indicates that glucose transport via GLUT3 is near maximal at normal plasma glucose concentrations [41]. GLUT1 and GLUT3 expression are regulated by developmental stage and by metabolic state.

Liver and pancreatic cells

GLUT2 is primarily expressed in hepatocytes and pancreatic β-cells with lower levels expressed in kidney and intestines. GLUT2 is a low-affinity receptor with a high turnover rate [41]. These kinetic properties allow GLUT2 to function in the liver where glucose transport must not be rate limiting for influx or efflux. When circulating glucose levels are high there needs to be net hepatic uptake as the intracellular glucose is metabolized or converted into glycogen. Conversely, when glucose levels are low, the liver needs to export glucose to the plasma. This is achieved by GLUT2 coupled with the regulated phosphorylating activity of hexokinase IV. Thus, during periods of glycogen synthesis hexokinase IV is up-regulated and increases the formation of glucose-6-phosphate [42]. This provides the precursor for glycogen synthesis and glycolysis and maintains intracellular glucose concentration low, which in turn drives the influx of glucose. In contrast, during glycogenolysis and gluconeogenesis, Hexokinase IV is down-regulated, intracellular glucose concentration becomes greater than in the plasma and there is a net efflux of glucose.

In order to regulate insulin secretion, pancreatic-cells need to be highly sensitive to changes in plasma glucose concentrations. Therefore, a low-affinity transporter, such as GLUT2, will not be saturated at physiological levels and glucose flux will be proportional to plasma glucose concentration. As in the liver, hexokinase regulates the entry of glucose into the glycolytic pathway and, along with GLUT2, plays a role in glucose sensing by β-cells [43-45].

Intestine and kidney

The small intestine and kidney express the isoforms GLUT1, GLUT2, GLUT3, GLUT5 and the Na^+-dependent glucose transporter. GLUT2 is the primary isoform responsible for transport across the basolateral membrane of intestinal epithelial cells [46] whereas GLUT5 mediates fructose uptake from the intestinal lumen and efflux from the intestinal epithelia [47]. GLUT2 can also transport fructose but with a six-fold lower affinity than GLUT5 [48]. Human digestive tract cancers (gastric and colorectal) show a distribution of GLUT over-expression with GLUT1, GLUT 2 and GLUT4 over-expressed.

Signaling pathways in GLUT regulation

Insulin pathway

Studies in adipose tissue, heart and skeletal muscle have shown that insulin-induced translocation of GLUT4 is mediated by phosphatidylinositol-3-kinase (PI3K) [49]. One possible mechanism for GLUT4 translocation is through the activation of protein kinase B/ Akt2, a downstream target of PI3K. Intracellular GLUT4-containing vesicles have a high basal level of PI4K activity. Insulin stimulation targets PI3K to these vesicles leading to the accumulation of these enzymes which act as docking sites for the recruitment and activation of Akt2. Akt2 phosphorylates vesicular proteins, including GLUT4, which causes the dissociation of the vesicles from an intracellular anchor and subsequent fusion with the plasma membrane [50]. Although the consensus is that PI3K is involved in insulin stimulated GLUT4 translocation, it is not clear whether other parallel pathways exist. There is evidence that the GTP-binding protein Gq can couple to GLUT4 translocation in adipocytes. This pathway is PI3K independent, requires tyrosine kinase activation and its inhibition prevents insulin-stimulated GLUT4 translocation [51] (Table 2). This data suggests that insulin causes GLUT4 translocation though at least two independent pathways in adipocytes. Ischemia, hypoxia and contraction cause GLUT4 translocation through a PI3K independent pathway [52-54].

A potential regulator of the pathway involved in metabolic stress-induced translocation of GLUT4 is AMP-activated protein kinase (AMPK) (Table2). Previous studies have shown that myocardial ischemia [55] and skeletal muscle contraction [56] activate AMPK and that activation of AMPK increases glucose uptake, which is not inhibited by wortmannin. Finally, it has been shown that AMPK activation causes the translocation of myocardial GLUT4 and increases glucose uptake [57].

Regulator	Pathway	GLUT Isoform	Cell type
Insulin	IR, PI3K	GLUT 4	Muscle, fat
IGF 1	IGF-IR, PI3K	GLUT 4	Muscle, fat
IGF 2	IGF-IR, PI3K	GLUT 4	Muscle, fat
Contraction	AMPK	GLUT 4, GLUT 1	Skeletal muscle
Ischemia	AMPK	GLUT 4	Heart muscle
Hypoxia	AMPK?	GLUT 4	Skeletal muscle
Nitric oxide	cGMP	Presumed GLUT 4	Skeletal muscle
Phorbol ester	PKC	Presumed GLUT 4	Skeletal muscle
a-Adrenergic agonists	Gs protein	GLUT 4	Brown fat, muscle
b-Adrenergic agonist	Gi protein	Presumed GLUT 4	Heart muscle
Bradikinin	Gq protein	GLUT 3	Skeletal muscle
Thrombin	Gi protein	GLUT 3	Platelets
Adenosine	Gq protein	GLUT 4	White and brown fat

Table 2 (adapted from [135]) - Regulatory and signaling pathways involved in regulation of GLUT protein in various tissues.

GLUT expression

Given the physiological importance of glucose uptake it is not surprising that GLUT expression is regulated, to some degree, by almost all of the know hormones. Insulin possesses long-term effects on GLUT content. Prolonged exposure to insulin, as occurs in type II diabetes, causes an increase in GLUT1 protein levels [58]. This is the result of enhanced GLUT1 mRNA transcription [59] and a rise in GLUT1 mRNA half life [60]. Nuclear hormone receptor ligands such as testosterone, glucocorticoids, retinoic acid and thyroid hormones have been shown to alter GLUT expression [61-64]. Further reports have demonstrated that prolactin [65], follicle stimulating hormone (FSH) [66], noradrenaline and the antidiuretic hormone, vasopressin [67] can also mediate expression.

Identification of new candidates in glucose transport

The success in identifying the intermediate steps between activation of insulin receptor and glucose transport has not been as rapid as that in elucidating the intermediate signaling events involved in stimulation of cell growth and mitosis.

In the case of the Ras/MAPK cascade a key switch in signaling occurs between tyrosine kinase activation and serine/threonine kinase activation. The switch occurs through the G-protein Ras that activates downstream Raf and mitogen activated protein (Map) kinases [68-71]. Such a switch has not been demonstrated for the reactions leading to stimulation of glucose transport. The divergence of mitotic stimulation and metabolic stimulation (via increased glucose transport) is clearly necessary in the insulin-target tissues of muscle and fat where increased metabolic flux must be acutely regulated over short timescales without a concomitant stimulation of cell growth and division. The extent to which these two signaling pathways diverge has also been difficult to dissect as studies of cell culture models and cell-free systems have suggested that there is a potential for cross-talk between these two pathways. Such crosstalk may be less significant in cells that are specialized to perform discrete functions. Given the complexity of the possible interactions between signaling intermediates, the mapping of a direct cascade leading to increased glucose transport is at present incomplete.

The cascades leading to stimulated glucose transport and activation of the Ras/MAPK pathway begin at the insulin receptor. A mutation in the ATP binding site of the tyrosine kinase domain of the beta subunit, leads to both defective activation of the Ras/MAPK pathway and defective stimulation of glucose transport [72, 73]. An active receptor tyrosine kinase is a prerequisite for GLUT4 translocation [74]. Taken together these data suggest that in signaling to downstream processes, the C-terminal autophosphorylation of the insulin receptor may be less important than the tyrosine-kinase-mediated phosphorylation of intracellular substrates. Further evidence for a divergence of signalling at the level of the insulin receptor comes from studies of a Tyr960-Ala mutation in the juxtamembrane domain. This mutant shows severely impaired tyrosine phosphorylation of IRS1 and Shc but only moderately impaired activation of Ras [75, 76]. The downstream activation of glucose transport is lost suggesting that this process is more associated with IRS1 and Shc than with activation of Ras [68].

IRS1, IRS2 and Shc are all phosphorylated in response to tyrosine phosphorylation by the insulin receptor. IRS1 also contains a phosphotyrosine binding (PTB) domain which recognizes the NPXY960 sequence of the insulin receptor. Mutagenesis has suggested that this region is important for IRS1 phosphorylation and activation of glucose transport. Glucose transport activity in IRS1-knockout mice is increased in response to insulin but the maximal stimulation is reduced in comparison with control mice [69, 70]. A complicating factor in interpreting these data is the presence of IRS2 in the IRS1-knockout mice [71]. IRS2 can also couple to p85 and may replace the missing signaling intermediate. Whether IRS2 plays a major role in normal stimulation of glucose transport in fat and muscle has not yet been fully evaluated.

Other evidence that IRS1 is utilized in initiating GLUT4 translocation has been obtained by antisense ablation of this molecule in rat adipocytes. The ablation results in a shift in the insulin dose response curve to the right (indicating a reduction in insulin sensitivity) but the maximal level of stimulation of transport activity is unaltered [76]. At present it is unclear whether IRS1 alone is responsible for downstream stimulation of glucose transport particularly as microinjection of anti-IRS1 antibodies, PTB domain constructs and NPX-phosphotyrosine peptides into 3T3-L1 adipocytes reduces IRS1 interaction with phosphatidylinositol (PI) 3-kinase but have no effect upon insulin-induced translocation of GLUT4. However, these treatments inhibit other aspects of insulin action including effects on membrane ruffling and mitogenesis [77].

Shc activation alone is insufficient to activate the MAP kinase pathway as the Tyr960 mutation of the insulin receptor leads to normal Shc phosphorylation but impaired signaling through Ras/MAP kinase. Since the Ras/ MAP kinase pathway does not appear to be involved in glucose transport stimulation, it seems unlikely that Shc is involved either. However, it cannot be ruled out that phosphorylated Shc may couple to pathways other than the MAP kinase pathway and that these do lead to glucose transport stimulation.

Hara et al. [78, 79] developed a mutant p85 which lacks a binding site for the catalytic p110 subunit [80]. This construct has been stably introduced into CHO cells overexpressing the insulin receptor. In this cell line, although activation of insulin receptor kinase and tyrosine-phosphorylation of IRS1 are unaffected, the insulin-induced increase in PI 3-kinase activity immunoprecipitated with anti-phosphotyrosine is greatly attenuated. Furthermore, insulin stimulation of glucose uptake is markedly impaired, in contrast to the insulin-stimulation of Ras activation, which is not. These results were the first to suggest that the p85 coupled PI 3-kinase is required for insulin-dependent glucose uptake [78, 79].

A strong correlation has been shown between the ability of wortmannin to inhibit PI 3-kinase and insulin-stimulated GLUT1 and GLUT4 translocation in 3T3-L1 cells. Both PI 3-kinase and glucose transporter translocation are inhibited by wortmannin in nanomolar concentration in 3T3-L1 cells [81]. Similar results have been found using LY294002 [82].
The discovery that PI 3-kinase is involved in insulin signalling to glucose transport stimulation has occurred at a time when there has been an accumulating body of evidence that the PI 3-kinase family of proteins is involved in a wide range of membrane trafficking processes. However, a major difference between the majority of the PI 3-kinase dependent membrane processes that have recently been described and the glucose stimulation effect is the magnitude of the response. These differences may be dependent on the type of PI 3-kinase involved in each of these processes, and the insulin-stimulated PI 3-kinase responsible for the 10-fold increases in glucose transport may involve a highly regulated form of PI 3-kinase. The intracellular membrane localisation of PI 3-kinase and the evidence that it may catalyze GLUT4 vesicle exocytosis suggest that it may act quite directly in the trafficking of GLUT4.

The physiologically important product of the action of the PI 3-kinase is thought to be PIP3. The PIP3 may interact with downstream signaling molecules and thereby transmit downstream the PI 3-kinase-dependent signaling processes, particularly since PIP3 is not a substrate for known phospholipases. There is evidence that PIP3 can interact with protein kinase B (PKB) [83] and with several protein kinase C (PKC) isoforms [84, 85]. There is a parallel translocation of PI 3-kinase and some of the PKC isoforms to the plasma membrane

in response to insulin [86, 87]. Presumably, the many downstream events mediated by PI 3-kinases are each catalyzed by many separate PIP3 binding proteins and therefore identifying the unique PIP3 receptor involved in regulating glucose transport may be a formidable task.

Serine and threonine kinases

Serine and threonine kinase action has been implicated in stimulation of glucose transport mainly because of the action of pharmacological reagents on this process. Among the list of reagents, okadaic acid (a protein phosphatase 1 and 2a inhibitor) and the PKC inhibitors polymyxin B and staurosporin have been studied most extensively. In most cases it has been unresolved where in the signaling cascade the protein kinase modifiers exert their effects. An effect of okadaic acid on the serine phosphorylation of IRS1 has been suggested leading to an inhibitory effect [88] while a direct stimulatory effect on translocation correlating with the phosphorylation of serine 488 of GLUT4 has been observed [89].

Phorbol-esters which stimulate PKC also lead to a stimulation of glucose transport activity [90, 91]. However, these effects are small in comparison with those produced by insulin [92, 93]. The effects appear to be due to 2–3-fold elevations in both GLUT4 and GLUT1 at the cell surface of rat adipose cells [92] and 3T3-L1 cells [93] whereas insulin produces 10–20 fold elevations of cell surface GLUT4. The effect may be due to stimulation of recruitment from an endosome pool that is common to GLUT1 and GLUT4 but is separate from the specialized pool from which insulin action is able to recruit GLUT4. PKC inhibitory agents such as polymixin B [94] and staurosporin [95] can completely inhibit insulin stimulated glucose transport but high concentrations of these reagents are required, and the specificity of the action of these compounds at these concentrations is unknown. Although the involvement of PKC isoforms in the stimulatory effects of insulin seems unlikely, alterations in insulin receptor tyrosine kinase activity mediated by PKC activity in response to elevations in cellular glucose may be an important feedback control of the signalling pathway [96]. The serine/threonine kinases which are downstream of Ras, those of the MAP kinase pathway, are unlikely to be involved in mediating the stimulatory effect of insulin on glucose transport. EGF and PDGF are able to mimic insulin's stimulatory effect on MAP kinase phosphorylation but do not mimic the stimulation of glucose transport [97, 98]. Birnbaum's group has convincingly shown that the activation of the serine kinase Raf (which is downstream of Ras and initiates the Map kinase cascade) does not lead to glucose transport stimulation. Expression of a constitutively active form of Raf in 3T3-L1 cells led to an increase in GLUT1 transcription but did not lead to the translocation of GLUT4 [99].

Insulin activation of glycogen synthesis involves direct phosphorylation of glycogen synthase kinase 3 (GSK3) by PKB and this occurs downstream of a wortmannin sensitive PI 3-kinase [100]. At present it is unclear whether PKB is so directly involved in regulating glucose transport. In 3T3-L1 cells expressing constitutively active PKB, glucose transport activity is elevated [101]. However, it remains to be established whether an inhibition of PKB blocks insulin stimulation of glucose transport. Activation of p70-S6 kinase appears to involve a divergent pathway from that leading to glucose transport stimulation. This pathway is wortmannin sensitive but is also rapamycin inhibitable, whereas rapamycin has no inhibitory effect on the pathway leading to insulin stimulation of glucose transport [102].

G-proteins

In 3T3-L1 cells that have been treated with wortmannin GTP-q-S is able to produce a stimulation of glucose transport. The effect is smaller than that produced in the absence of the PI 3-kinase inhibitor but this result suggests that G-proteins may be downstream targets of PI 3-kinase in the cascade of intermediates leading to glucose transporter translocation to the plasma membrane [103]. This type of experiment does not distinguish between an involvement of trimeric G-proteins and those of the Ras/ Rab/Arf super family of small (» 20 kDa) G-proteins. Indeed, although the small G-protein family members seem more likely targets of PI 3-kinase signalling, the trimeric G-protein Gαi is strongly implicated in the regulation of glucose transporter translocation since a knock-out of this protein in transgenic mice completely blocks the insulin stimulation of GLUT4 translocation [104]. These studies conclude that Gαi is an early signaling intermediate above the level of IRS1 phosphorylation. However, when rat adipose cells are treated with insulin and then isoproterenol, which activates Gαi, they have an attenuated stimulation of glucose transport activity compared with insulin alone. It has been postulated that this effect occurs at a Gαi-dependent GLUT4 vesicle fusion step, a late effect in the cascade linking insulin and glucose transport [105]. Further experiments will presumably clarify the level (or levels) at which Gαi acts.

The GTPase dynamin is important for vesicle budding processes. Interestingly, dynamin in a complex with Grb2, can be immunoprecipitated with IRS1 in insulin-treated CHO-IR cells [106]. This observation may be significant as it is an additional point of convergence of signaling and trafficking processes. The relationship of this coupling to insulin-stimulated glucose transport has yet to be investigated. The small G-protein Ras appears not to be involved in normal insulin signalling to glucose transport as the dominant-negative Ras [107] or Ras neutralizing antibodies [108] do not inhibit glucose transport under conditions where the activation of the Map kinase cascade is blocked. There are now examples of small G-

proteins acting downstream of PI 3- kinase in signalling cascades. Rac has been shown to be involved in the membrane ruffling process that is downstream of PI 3-kinase. Modulation of GTP exchange on Rac is postulated to be the means by which its activity is regulated [109]. Insulin stimulated membrane ruffling is inhibited by the D-p85 construct [110] and by dominant negative Rac. However, Rac does not appear to be involved in the stimulation of glucose transport in 3T3-L1 cells as the introduction of a dominant negative construct of Rac does not significantly inhibit insulin action on this process [111].

Attractive alternative candidates as the downstream G-proteins in insulin action are the Rab proteins. Although the suggestion that Rab4 is present on GLUT4 vesicles [112] has been disputed, it is clear that perhaps unidentified small G-proteins of this family are present [113].

The case in Drosophila

Glucose/Sugar transporters in Drosophila

Very little is known about the cellular sugar transport systems in Drosophila. The principle circulating sugar in Drosophila haemolymph is Trehalose, which is discussed below in detail. Drosophila genome contains several genes which are predicted to have sugar or glucose transport functions [FlyBase]. They consist of four sugar transporter genes, Sut 1-4. Sut 1-3 are clustered at position 44A4, while Sut 4 exists at 46E-4. Two genes encoding for proteins similar to Glucose transporters have been identified in Drosophila genome; namely Glut 1 and Glut 3. BLAST shows that Glut 1 of Drosophila shares homology with human Glut 1-4, and Glut 3 shows weak homology with human Glut 6 and Glut 8. It is not clear what functions these putative sugar and glucose transporters might be having in Drosophila. In addition, there are around 9 more genes which are predicted to encode for sugar transporters. Hence at this point, it is difficult to predict which of the sugar transporters in Drosophila might be playing a role, if at all, in the uptake of Glucose, and if any of these sugar transporters could be regulated by insulin signaling.

Trehalose

Trehalose is a disaccharide composed of two glucose molecules bound by an alpha-1, 1 linkage. Resistance to acid hydrolysis and an absence of direct intramolecular hydrogen bonding make trehalose chemically unique when compared with other common disaccharides, particularly sucrose, the non-reducing disaccharide of plant origin. Trehalose is widely distributed in nature. It is known to be one of the sources of energy in most living organisms

and can be found in many organisms, including bacteria, fungi, insects, plants, and invertebrates. Synthesized in the fat body following digestion of dietary sugar, trehalose is a condensation product of two glycotic intermediates, glucose-1-phosphate and glucose-6-phosphate. Alternative sources of trehalose are glycogen breakdown. Trehalose synthesis and degradation are under hormonal control involving both hypertrehalosmic and hypotrehalosmic factors. Trehalose concentration in the haemolymph, however, is not homeostatically regulated. Rather, trehalose occurs at highly variable levels, typically between 5 to 50mM, depending on environmental conditions, physiological state and nutrition. This variable concentration is essential for fulfilling the roles of trehalose, as 1) an energy store, the traditional role ascribed to trehalose, 2) a cryoprotectant, reducing the supercooling point of some freeze-avoiding insects; 3) a protein stabilizer during osmotic and thermal stress, a function only recently investigated in insects 4) a component of a feedback mechanism regulating feeding behavior and nutrient intake, where blood metabolite levels influence food selection through the central nervous system.

Trehalose, however, is only one of the many metabolites occurring at high concentrations in insect blood. Glucose is often present, sometimes at levels similar to those of mammalian blood, but generally lower than trehalose. Amino acids, proteins and other organic compounds are also present, and their concentrations may exceed that of trehalose.

Trehalase

Trehalase catalyzes the irreversible hydrolysis of trehalose to glucose. As the only known enzyme for metabolizing trehalose to glucose, it is not surprising that trehalase activity is found in many forms and is widely distributed among insect tissues and organs including haemolymph. Numerous membrane-bound and soluble isozymes have been isolated. The kinetics and conditions for optimal activity vary greatly between enzymes from different insects and tissues. Trehalose hydrolysis is perhaps best understood in asynchronous flight muscle of dipteran insects, many of which utilize trehalose as the principal source of energy during flight. In *P.regina*, the enzyme is localized in the inner membrane of the mitochondria, and there is a strong relation between wing beat frequency and decreased haemolymph trehalose level. Trehalase activity is present at relatively high levels in the insect midgut. There, it may aid in maintaining the glucose concentration gradient across the midgut epithelium, by hydrolyzing trehalose, and preventing the diffusion of trehalose from haemolymph into the gut lumen.

Hypertrehalosmic factors

Steele [115] was the first to demonstrate that extracts of the corpora cardiaca injected into the American cockroach stimulate glycogen breakdown and release of trehalose from the fat body into the haemoplymph. By comparison with glucagons, Steele [116] suggested the name trehalogon for the factors responsible, but they are generally known as hypertrehalosmic hormones. They are octa-, nona-, and decapeptides with several common structural features necessary for activity [117-120]. The hormones do not always produce an elevation in haemolymph trehalose level, but those that affect carbohydrate metabolism all act by stimulating glycogen breakdown [120]. In some insects, for example in locusts, the same hormone acts to stimulate lipolysis, the hydrolysis of triglycerides, in addition to glycogen breakdown. Thus the peptides are also known as adipokinetic hormones [121]. Some insect species have more than one hormone. The hypertrehalosemic factor and adipokinetic hormones of locusts and cockroaches are released from the corpora cardiaca upon initiation of and during exercise and flight [121, 117]. They act to provide substrate, glucose and/or fatty acids, for supporting aerobic respiration.

Two studies have been published where the Drosophila *Akh* gene has been characterized. Park et al. show that *Akh* expressing neurons are present exclusively in the corpora cardiaca in the larval and adult stages. Projections emanating from the AKH neurons have multiple target tissues as follows: the prothoracic gland and aorta in the larva and the crop and brain in the adult. Studies using transgenic manipulations of the *dAkh* gene demonstrated that AKH induced both hypertrehalosemia and hyperlipemia. Additionally Rulifson et. al. showed that *Akh* expressing cells in corpora cardiaca also express Drosophila cognates of sulphonylurea receptor (Sur) and potassium channel (Ir), proteins that comprise ATP-sensitive potassium channels regulating hormone secretion by islets and other mammalian glucose-sensing cells. They also show hyperglycaemia upon ablation of these neurons and suggest that AKH might have functions similar to Glucagon.

Hypertrehalosemic hormones stimulate glycogen hydrolysis in a similar manner as the mammalian hormones glucagons and epinephrine. They act on the protein kinase enzyme cascade that activates phosphorylase kinase, and ultimately glycogen phosphorylase [121, 122]. The activities of hypertrehalosemic and adipokinetic hormones are mediated through several signal transduction pathways. In locusts, hormone-stimulated glycogenolysis involves both a cAMP/G protein coupled pathway [124, 125] as well as extracellular and intracellular Ca^{2+} [136]. Hypertrehalosemic action in cockroaches, however, is not mediated through cyclic

nucleotides [127]. These differences between species occur despite the structural similarities of the hormones involved [128].

Hypotrehalosemic hormones

Insulin

Insulin in mammals induces cells to take up glucose and convert it to glycogen, to inhibit glycogen breakdown and gluconeogenesis, and generally to shift from catabolic to anabolic lipid and protein metabolism. As a test of the concept that insulin structure and function are conserved, most early studies focused on eliciting these regulatory effects in insects with vertebrate insulins or in mammalian bioassays with purified insect Insulin-like peptides. Now with the identification of several insect ILPs, this concept awaits validation by testing synthetic ILPs for direct effects in bioassays, and to date, only two such studies exist. When injected into neck-ligated *B. mori* larvae, Bombyxin II lowered the concentration of trehalose, the major haemolymph sugar, and induced elevated trehalase activity [129]. The injected ILP also lowered glycogen content and raised glycogen phosphorylase activity in the fat body. The apparent role of the injected ILP in *B. mori* larvae was to promote consumption of carbohydrate reserves and not the accumulation of reserves, as for insulin in mammals. When injected into adult *B. mori*, this ILP, however, had no effect on trehalose or lipid levels in haemolymph [130]. Contrasting results for an ILP administered to different life stages of an insect suggest that ILP regulation of metabolism may be particularly complex. In Drosophila, targeted expression of cell-death-promoting factors to ablate brain mNSCs that express DILPs elevated trehalose and glucose levels in the haemolymph [131, 132]. Although there is no significant difference in levels of proteins and glycogen, dwarf flies with mutations in *dinr* and the *chico* gene exhibit up to a twofold increase in lipids [133]. Although homologous genes for glucose transporters and enzymes involved in glycogen synthesis have been identified in Drosophila, only one study has looked at glucose uptake and metabolism. Insulin had no effect on glucose uptake or lipid synthesis and reduced glycogen in Kc cells, as above with silkmoth larvae, but it did increase glucose oxidation and lactate production, thus providing biosynthetic precursors needed for growth [134].

It is not clear whether Insulin plays a direct role in maintaining haemolymph sugar levels. Also, since there are a few DILPs expressed in Drosophila, it is possible that some of them are specialized in maintaining trehalose levels. Hence, I tried to establish assays to measure any effect of Insulin on sugar uptake in cell culture and on tissues.

For this purpose all three cell lines available in our lab, Kc, S2 and Clone 8, were tested. The protocol followed for establishing glucose uptake assays was obtained from the group of Markus Niessen, where glucose uptake assays are done routinely using differentiated adipocytes. For assays on tissues, various tissues were dissected from wandering third instar larvae. Preliminary experiments were performed using 0.1% glucose containing medium for starvation of cells. Later medium without glucose was used for starvation of cells and tissues.

Materials and Methods

Glucose uptake by Clone 8, Kc 167 and S2 cells

The experiments were performed in 6 well plates (for adherent Clone 8 cells) or in eppendorf tubes (for non-adherent S2 or Kc cells). 3-5 million cells per sample were collected (S2 and Kc cells) by centrifuging at 2600 rpm for 5 min at room temperature and washed twice with serum and glucose-free DMEM medium (Invitrogen, DMEM, 1X, liquid without glucose, Catalog no.- 11966025). The cells were starved in 1 ml of this medium for 1 hour. Various concentrations of DILP 2 (tested in all cell lines), 3, 4 or 5 (tested on S2 cells) and 100nCi of D-[U-^{14}C] Glucose (Amersham, Catalog no- CFB2), diluted in DMEM medium, were given to these cells and mixed gently. After incubation for 30 minutes at room temperature, the cells were placed on ice. All the following steps were performed at 4°C. The cells were centrifuged down at 2600 rpm for 5 min, supernatant was discarded and cells were washed three times with ice cold PBS. Finally, cells were resuspended in 100 µl RIPA lysis buffer with protease inhibitors, and shaked gently for 15min at 4°C. 50 µl of the lysate (taken without centrifugation), were placed in 6 ml plastic scintillation vials (Millipore). 2 ml of BCS Scintillation cocktail (Amersham, Catalog no- NACS204) was added to each vial. The sample cpm was read in a beta-Scintillation Counter (Beckman Coulter, RP1000) with settings of C-14 (1 min. measuring time). The specific activity of the D-Glucose stock was determined in each experiment by using 1% of the C-14 labeled glucose. A blank vial was also included in each case. Non-DILP-stimulated and non-starved cells were used as controls. For normalization, total protein concentration of each sample was determined using Bradford assay (RC DC kit, Amersham). CPM values of each sample were normalized to counts per 100 µg of total protein. Average, standard deviation and significance were determined. Data is represented as histogram plots. In case of Clone 8 cell line (adherent cells), all the washing and lyses were performed in the six well plates, rest of the procedure remained the same. In some cases, Wortmannin or Cytochalasin D was used at final concentrations of 10nM each.

Glucose uptake assays by third instar larval tissues

Various tissue types of the wandering third instar larvae were isolated. These were muscles (mostly bound to the cuticle), soft tissues (all tissues, without cuticle), fat body and whole larvae (cuticle and all tissues). 20 larvae per sample were used. Tissues were dissected in PBS and immediately placed in starvation medium (DMEM medium without glucose). Tissues were starved for 1 hour before addition of DILP and 100 nCi of D-[U-^{14}C] Glucose. The tissues were incubated for 30 minutes at room temperature and centrifuged to remove the supernatant. The samples were washed three times with ice-cold PBS and resuspended in 100 µl lysis buffer for 1 hour at 4 °C. Remaining intact tissue was crushed with the help of a pestle. 50 µl of the lysate was used to measure the cpm, and the rest of the procedure was same as above.

Results and Discussions

Insulin has been shown to promote the uptake of glucose by certain cell types, which have been deprived of glucose. Although this effect of insulin is very well established and is very frequently used as readout of its activity in mammalian systems, very little is known about the genes involved in this effect of insulin (as discussed in Introduction). Several research groups are trying to find genes that bring about this effect of insulin. Most of these studies are done using specialized cell lines like 3T3-L1 cells, which can be differentiated into adipocytes, or on freshly dissected tissues such as rat muscles or adipose tissues. The use these cell lines or tissues in order to find new genes trying to in glucose uptake has its limitations.

The Insulin pathway in Drosophila bears similarities to the mammalian Insulin/IGF signaling pathway, and growth effects of this pathway are characterized to a good extent. However, the metabolic effects of Insulin in Drosophila are not well addressed. There could be several advantages of using Drosophila and Drosophila cell culture as a model system in studying the metabolic effects of insulin like, for e.g., easy availability or ease of creating mutants and ease of RNA interference in cells lines. Keeping this in mind it seemed worthwhile to establish such an assay in Drosophila. The second reason why establishing glucose uptake assays in Drosophila seemed important is the presence of seven putative Insulin-like peptides. Like in humans, the IGFs regulate the growth, and insulin the glucose levels, testing their bioactivities would allow to establish whether such a distribution of function is present in Drosophila too.

In glucose uptake assays on adipocytes, the cells are first starved for glucose using a low (0.1%) D-Glucose containing medium. This low amount of glucose present in the starvation medium prevents the cells from dying due to complete lack of glucose. For preliminary experiments, the same DMEM medium with low glucose was used for Drosophila cells as well. However, using this medium for starvation gave highly variable and inconclusive results. Hence, I used a medium which lacked glucose completely. Drosophila cells can survive well in complete lack of glucose for at least 2 hours.

Experiments using '0.1% glucose containing' starvation medium

Figure 1b shows the amount of glucose (measured as beta counts per minute per 100ug of total protein) taken up by Kc cells after one hour of starvation in serum free, 0.1% glucose containing medium and then stimulated with various concentrations of DILP 2. There seems to be no effect of DILP 2 on these cells in terms of taking up glucose. In general the uptake under these conditions seemed to be very low as compared to uptake by adipocytes under similar conditions (Figure 1a). Clone 8 (Fig 1c) cells similarly showed low counts, large deviations and no clear effect of insulin on the uptake. Taken together these cell lines did not give any conclusive results on the glucose uptake. However, S2 cells, which otherwise did not show high counts, did show slight increase in uptake with increasing concentrations of DILP 2, up to a concentration of 50nM. However, at a concentration of 100 nM the counts were again low. This indicated that DILP 2 at medium and high concentrations can stimulate uptake, but with excessively high concentration the whole system is shut down and cells do not take up glucose anymore. Hence for further experiments, S2 cells were chosen. These preliminary experiments were done using only DILP 2 and only in triplicates per concentration used.

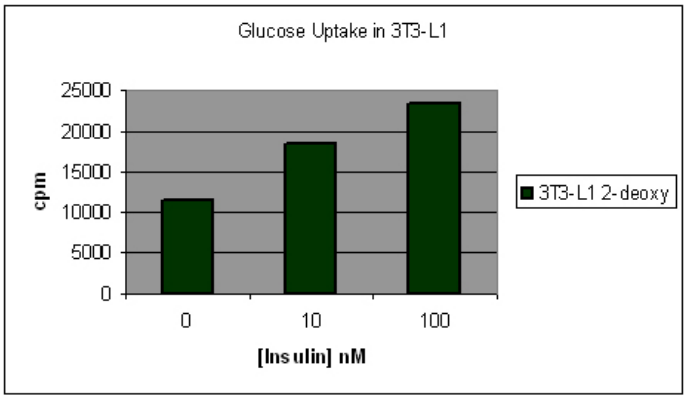
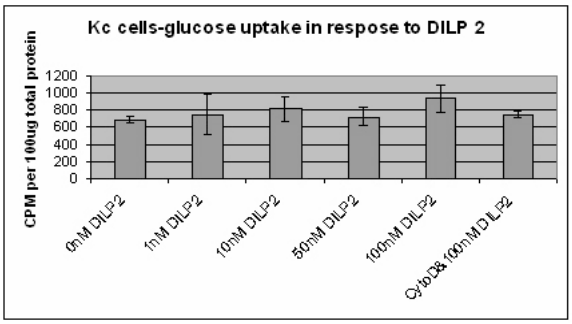
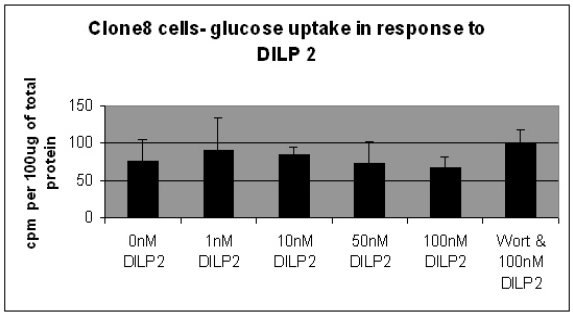


Figure 1a shows the uptake of Glucose by 3T3-L1 cells when stimulated with increasing concentrations of Insulin. 3T3-L1 cells which were induced to differentiate into adipocytes were starved with 0.1% Glucose containing DMEM. The cells were then induced with human Insulin at 10nM and 100nM concentrations. After one hour incubation the cells were washed, lysed and the lysate was counted for CPM. The cells show an increase in the uptake of

Glucose with increasing concentrations of Insulin. It is noteworthy that these cells take up substantial amount of Glucose even without stimulation with Insulin.





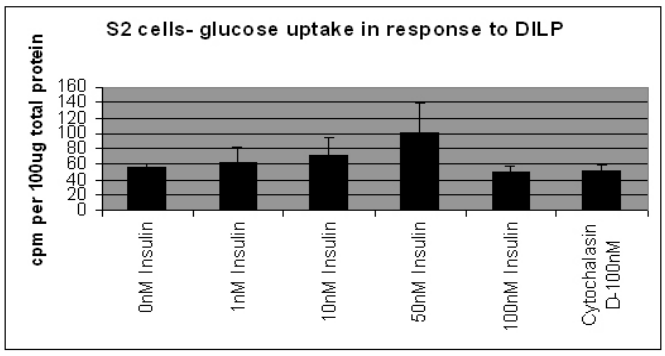
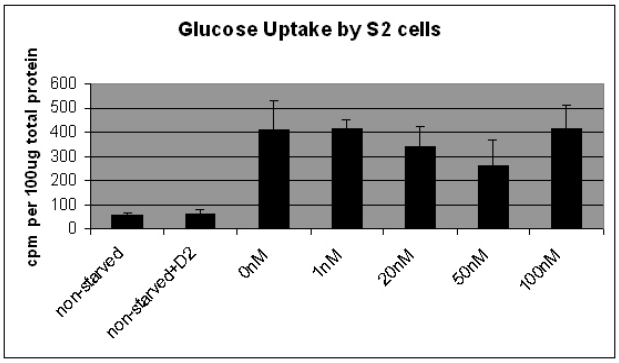


Figure 1b-d show Uptake of glucose by S2, Kc and Clone 8 cells upon stimulation by various concentrations of DILP 2. Inhibitors like Cytochalasin D or Wortmannin were used to test if they have any negative effect on Glucose uptake. These preliminary results do not show any stimulatory effects of DILP 2 on the uptake of glucose by the cell lines.

Experiments using starvation medium without glucose

All the experiments using glucose-free starvation medium were done on S2 cells using 8 samples per concentration of DILPs tested. These experiments have shown higher counts and more consistent results. The readings have been higher most probably due to complete lack of glucose in the starvation medium. Fig 2a clearly shows that as compared to unstarved controls including DILP 2 stimulated and unstimulated cells, S2 cells took up glucose upon starvation with a glucose free medium. However, addition of DILP 2 did not have a significant effect on this uptake of glucose. It can be concluded that Drosophila cells do take up glucose upon starvation for this sugar. However, stimulation by DILP 2, under these conditions, doesn't affect the uptake of glucose. Stimulation by other DILP 3, DILP 4 and DILP 5 also do not show any effect on the uptake of glucose by Drosophila cells, under the conditions tested (Fig 2b). In case of these DILPs, a single concentration, at which they show maximum phosphorylation of S6 kinase, was tested.



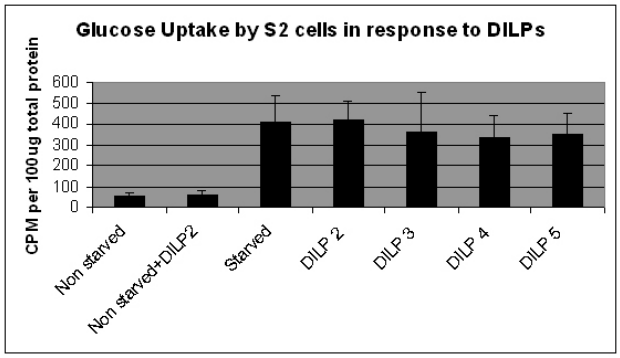


Figure 2 a and b show uptake of Glucose by Drosophila S2 cells. S2 cells starved with glucose-free DMEM medium take up substantial amount of glucose as compared to cells placed in normal DMEM medium or normal medium stimulated with DILP 2. However, stimulation with DILP 2 at various concentrations does not show any consistent and clear increase in the uptake of glucose.

Glucose uptake by tissues

In mammals, the muscles and adipose tissue are specialized in the uptake of glucose. Insulin is one of the major regulators of uptake of glucose in these tissues. In Drosophila, fat body has been shown to be an important storage tissue and has humoral functions associated with nutrition, comparable to vertebrate liver and adipose tissue. During larval stages, the fat body accumulates large stores of proteins, lipids, and carbohydrates, which are normally degraded by autophagy during metamorphosis in order to supply the developing tissues [136], but can also be remobilized during larval life to compensate transitory nutrient shortage. In addition to its storage function, the fat body also has endocrine activity and supports growth of imaginal disc explants and DNA replication of larval brains in co-culture experiments [137, 138]. In 2003, Colombani et al. demonstrated that the Drosophila fat body operates as a sensor for variations in nutrient levels and coordinates growth of peripheral tissues accordingly via a humoral mechanism. Whether insulin signaling in Drosophila can directly stimulate fat body or other tissues to take up and store sugar is not clear. Hence, I performed preliminary glucose uptake assays on various tissues of the third instar larval stages.

Preliminary experiments were performed on various tissues of third instar larva using 0.1% starvation medium. The experiments were done using various tissues which included whole soft tissues (all tissues except cuticle), fat body and muscles. The data obtained using 0.1% glucose containing medium were inconclusive mainly because the counts were low and inconsistent (data not shown). Furthermore, the handling of fat body proved to be difficult. Hence larval muscle was chosen for further experiments using glucose-free starvation

medium. Starving third instar muscle tissues with a medium lacking glucose completely stimulated the muscle cells to take up glucose in significantly higher amounts as compared to non-starved muscle cells. Stimulation by DILP 2, 3, 4 and 5 did not lead to any increase in the uptake of glucose by these muscle tissues.

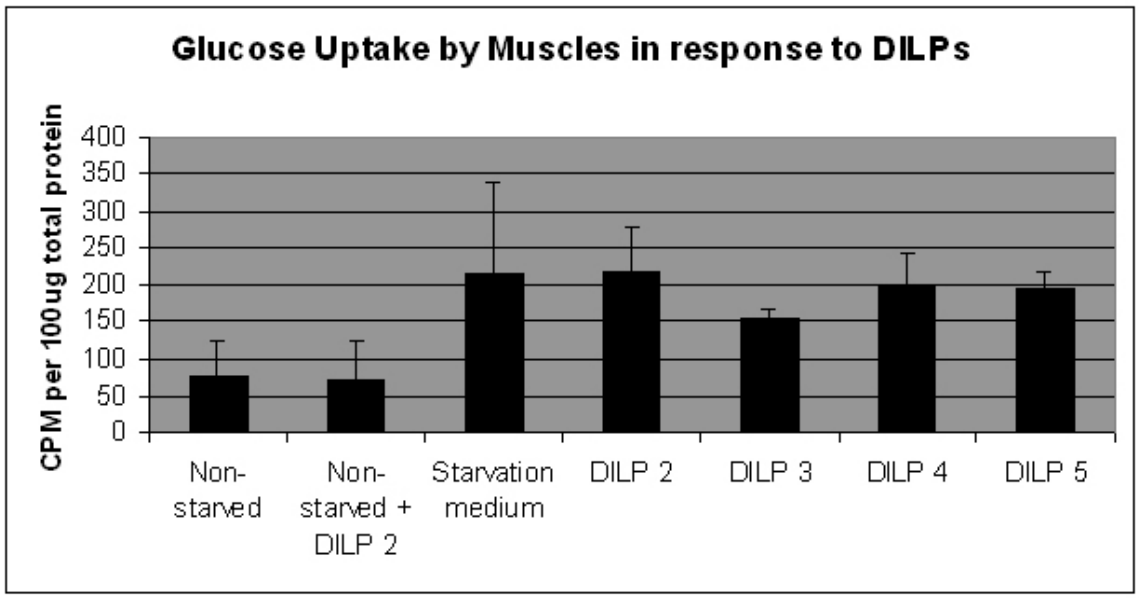


Figure 3 shows glucose uptake by muscles in response to stimulation by various DILPs, after starvation in glucose-free media, compared to muscles incubated with glucose-free DMEM only or normal DMEM with DILP 2 only. The muscles take up glucose upon starvation but stimulation by various DILPs do not seem to have a stimulatory effect on it.

Taken together, the results indicate that DILPs do not stimulate glucose uptake under the conditions tested. This however does not exclude that DILPs play a significant role under normal physiological conditions in Drosophila. Depriving Drosophila cells or tissues completely of glucose created an unnatural condition. Under such conditions the cells might simply take up glucose and do not need stimulation by factors like Insulin. Under more physiological conditions insulin signaling might be playing a role in regulation of haemolymph sugar levels. However, it was not possible to simulate exact physiological conditions in these assays.

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CHAPTER 5

Molecular characterization of the insulin pathway in Drosophila using RNA interference

Introduction

The existence of seven putative Insulin-like peptides in Drosophila raises the obvious question- whether they all bind to and signal through the single Insulin receptor homolog present in Drosophila. As discussed in previous chapters, there is evidence in mammalian systems that Insulin-like peptides can bind to receptors completely different from Receptor tyrosine kinases. For example, relaxin, which is structurally very similar to Insulin, binds to and activates a GPCR.

This may well be possible for some of the DILPs. There have been no studies in this direction in Drosophila so far, and there is no evidence to suggest that some of the DILPs might have other receptors. When the DILPs are overexpressed, they all lead to body size increase although to various extents. This might suggest that these peptides are able to bind to the single insulin receptor, but it is possible that some of these peptides are binding to other receptors and can have completely different physiological functions.

This question can be addressed by down regulating certain receptor candidate homologs in cell culture and checking if this results in Insulin activity. However, this approach relies on robust readouts.

I wanted to address this question to some extent using the purified DILPs, but was restricted by the readouts of insulin activity available in the Drosophila system. The DILPs did not stimulate uptake of glucose by Drosophila cells upon stimulation with various DILPs. Effects on growth and proliferation were being established. Hence, I decided to choose phosphorylation of downstream kinases of the InR as readouts of insulin activity. I knocked down the receptor in cell culture using RNA interference and then observed the phosphorylation on downstream kinases like PKB and S6Kinase. This could at least establish whether dInR is the sole signaling receptor to S6Kinase and PKB. In the process, I characterized the DILP signaling pathway molecularly in cell culture using RNAi [1].

The insulin signaling pathway is described in detail in Chapter 1. Recently, two new additions have been made to this pathway.

Ma et al. [2] showed that Erk dependent phosphorylation leads to TSC1-TSC2 dissociation and markedly impairs TSC2 ability to inhibit mTOR signaling, cell proliferation, and oncogenic transformation. Raf-MEK1/2-Erk1/2 signaling cascade leads to the phosphorylation of TSC2 by Erk1/2 and the consequent functional inactivation of the TSC1-TSC2 complex. This signaling axis can therefore cooperate with the PI3K/Akt axis in inactivating TSC2 through phosphorylation on distinct residues, thus leading to mTOR activation, S6K activation, 4E-BP1 inactivation, and ultimately, activation of mRNA translation.

Akt/PKB activation requires the phosphorylation of Thr308 in the activation loop by the phosphoinositide-dependent kinase 1 (PDK1) and Ser473 within the carboxyl-terminal hydrophobic motif by an until recently unknown kinase. Sarbassov et al [3] showed that in Drosophila and human cells the target of rapamycin (TOR) kinase and its associated protein Rictor are necessary for Ser473 phosphorylation and that a reduction in rictor or mammalian TOR (mTOR) expression inhibited an Akt/PKB effector. The Rictor-mTOR complex directly phosphorylated Akt/PKB on Ser473 in vitro and facilitated Thr308 phosphorylation by PDK1 [3]

Materials and Methods

RNA interference

dsRNA targeting of components of Insulin-TOR pathway was done using short dsRNA synthesized by in vitro transcription in 20 µl reactions using a T7 MEGAscript™ kit (Ambion). The primer sequences (which incorporated a 5' and 3' T7 promoter) of various pathway components used for PCR fragment generation are given below

dInR, forward primer – cgcTAATACGACTCACTATAGGGAGAgtctttcaacaacatctgtatggactc
dInR, reverse primer – cgcTAATACGACTCACTATAGGGAGAgactcgaatggaaaccgcggatca
dMEK, forward primer - agTAATACGACTCACTATAGGGAGAatcaagatgttcctcagc
dMEK, reverse primer - agTAATACGACTCACTATAGGGAGAttgggcggcggttcgtttaca
dTOR, forward primer - agTAATACGACTCACTATAGGGAGAtgagctggctatcgccttgc
dTOR, reverse primer - agTAATACGACTCACTATAGGGAGAgcggtgcagagtgcgatcgaa
Class I PI3K, forward - acTAATACGACTCACTATAGGGAGAtacgagctgaccaagatggttaacg
Class I PI3K, reverse - acTAATACGACTCACTATAGGGAGAtcaactgaaagtagttgcccatgtc

Class II PI3K, forward - aTAATACGACTCACTATAGGGAGAagtccgaatgttggtctaatcggttg
Class II PI3K, reverse – acTAATACGACTCACTATAGGGAGAaatgggcttgaccgcataatctgtg
Class III PI3K, forward –acTAATACGACTCACTATAGGGAGAatcaagagcgataagtcagtgagtc
Class III PI3K, reverse – acTAATACGACTCACTATAGGGAGAtactcgtgatgggcaatcgaagtg
Lgr3, forward (1) - acTAATACGACTCACTATAGGGAGA atgcgagatatccgccttgtcgg
Lgr3, reverse (1) – acTAATACGACTCACTATAGGGAGActccatcggtggacggcttgcac
Lgr3 PI3K, forward (2) – atTAATACGACTCACTATAGGGAGAaaggagattgccgacaagattgtcc
Lgr3 PI3K, reverse (2) – acTAATACGACTCACTATAGGGAGAttctgcagacgcgtgaaattggc
dPKB, forward – aTAATACGACTCACTATAGGGAGAagtccgaatgttggtctaatcggttg
dPKB, reverse- acTAATACGACTCACTATAGGGAGAaatgggcttgaccgcataatctgtg

Kc 167 cells were grown to 60-80% confluence in Schneider's medium (Invitrogen, Catalog no. - 11720-034) with 5% fetal calf serum (Invitrogen, Catalog no.- 16000-044) and penicillin/streptomycin, and dsRNA was added according to the protocol from Ambion. dsRNA containing medium was left on the cells for 7 days before stimulation by DILP 2.

DILP stimulation and Western blotting

Inhibitors like Wortmannin (Biochemika, catalog no. - 95455), LY-165,163 (Sigma, catalog no. – S0009), PD-098059 (Sigma, catalog no. – P3209), U0126 (Sigma, catalog no. – U120) were used at a concentration of 100 μM, 1 hour before DILP stimulation. Activators like Forskolin (Sigma, catalog no. – 6886) was used at a final concentration of 100 μM for one hour before DILP 2 stimulation. After 20 min. stimulation the cells were placed on ice and collected by centrifuging at 300 xg for at 4°C 5 minutes. The supernatant medium was discarded and the pellet was washed by cold PBS. Cells were lysed by lysis buffer (120mM NaCl, 50mM Tris-HCl, 20mM NaF, 1mM Benzamidine, 1mM EDTA, 6mM EGTA, 15mM NA_4P2O_7, 1% Nonidet P-40) containing protease inhibitors like 1x Mini Cocktail Tablets (Roche, catalog no.- Cat. No. 04 693 159 001) and phosphatase substrates like pNPP (Fluka, Catalog no. -71770) and beta-Glycerol phosphate (Sigma, G 6251). The cells were shaken in lysis buffer at 4°C for 15 minutes and lysate was centrifuged at 4°C at 13,000 rpm for 15 min. The supernatant was collected and total protein concentration was estimated. 100 μg of total protein of lysate was heated with 4x Loading buffer (0.5M Tris HCl, 1M DTT, 20% SDS, 10% Glycerol, 0.2% Bromophenolblue) and loaded on a 10% SDS PAGE gel. The proteins on gel were blotted onto a Nitrocellulose (Amersham, Hybond™ ECL™, catalog no.- RPN2020D) membrane overnight at 4°C at 150mAmp. Thereafter, the membrane was blocked with 3% ECL blocking powder (Amersham, catalog no.- RPN418). Primary antibodies like anti phospho Akt (Ser 473) (Cell Signaling, catalog no.- 9271S), anti phospho-

p70 S6 kinase (Thr 389) (Cell Signaling, catalog no.- 9205S), anti dInR (kind gift from Rafael Fernandez), total PKB (Cell signaling, catalog no. - 9272), total p70 S6 kinase (Cell signaling, catalog no. -9202), anti-MEK (Cell signaling, catalog no. - 9124), and anti-tubulin (Sigma, catalog no- T9026) were used at 1:1000 and 1:100,000 respectively. Secondary antibodies like anti-rabbit HRP (Jackson Immunoresearch, catalog no. - 111-035-003) and anti- mouse HRP (Amersham, NA931VS) were used at 1:10,000 dilution. The blots were developed using ECL Western Chemiluminescence Kit (Amersham, catalog no. - RPN2109).

Results and Discussion

DILP 2 signals to S6 Kinase solely via dInR and dPKB

dInR was knocked down in Kc cells by using RNA interference, and phosphorylation of S6 kinase and PKB upon DILP 2 stimulation was checked using antibodies recognizing phosphorylated serines or threonines. When dInr was knocked down, no activation of dPKB was observed, but there was a slight activation of dS6 kinase, which was clearly above its basal level of activation (Fig 1a). Anti-InR antibody showed robust downregulation of the insulin receptor in cells with RNAi (Fig 1a). This gave rise to many questions: Is PKB is really not involved in the slight activation of S6 kinase? Or is the signaling mediated by PI3K and TOR but independent of PKB? Or are other receptors involved in signaling to S6 kinase in response to DILP 2? When considering other candidate receptors, I chose to downregulate dLgr3. dLGR3 is the single homolog of human LGR7 and LGR8 in Drosophila, which codes for a G-protein coupled receptor. Hsu et al. [4] showed that LGR7 and LGR8 are capable of mediating the action of Relaxin through an adenosine 3', 9'-monophosphate (cAMP) dependent pathway distinct from that of the structurally related insulin and insulin-like growth factor family ligand. Hence, dLgr3 was the first receptor candidate that I checked.

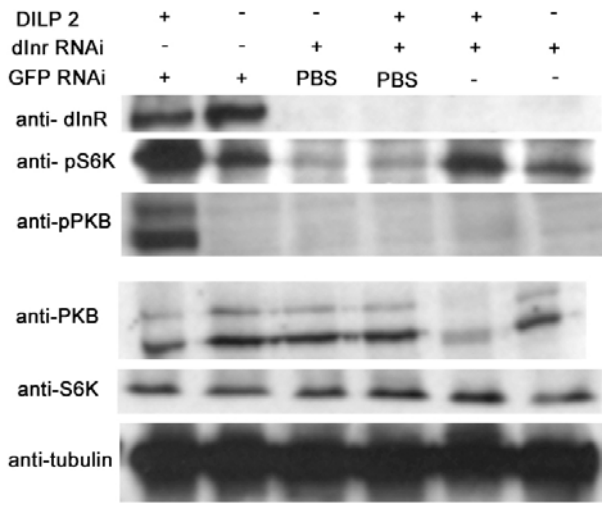


Figure 1a shows phosphorylation of S6 kinase and dPKB upon stimulation of Kc cells, which were treated either with dsRNA for dInR or GFP, with DILP 2. Lanes marked with 'PBS' are lysates of cells, stimulated with DILP 2 in PBS instead of stimulating in normal Schneider's medium. D2 is DILP 2.

Lane 5 (from left) shows phosphorylation of S6 kinase but no phosphorylation of PKB can be seen.

In the same experiment, I also used Wortmannin, an inhibitor of all classes of PI3 kinase [5]. Preliminary experiments by knocking down dLgr3 with RNAi showed that the slight activation of S6 kinase seen when dInR is downregulated could be dependent on dLgr3 (Fig 1b). The slight signal of S6 kinase activation was gone when Lgr3 was downregulated. This also seemed to be Wortmannin sensitive and thus involvement of PI3 kinase seemed possible. This was plausible since GPCR are known to activate PI 3 kinases [6]. However, the use of Wortmannin does not allow deciphering which class PI3K could be involved in the extra activation of S6 kinase.

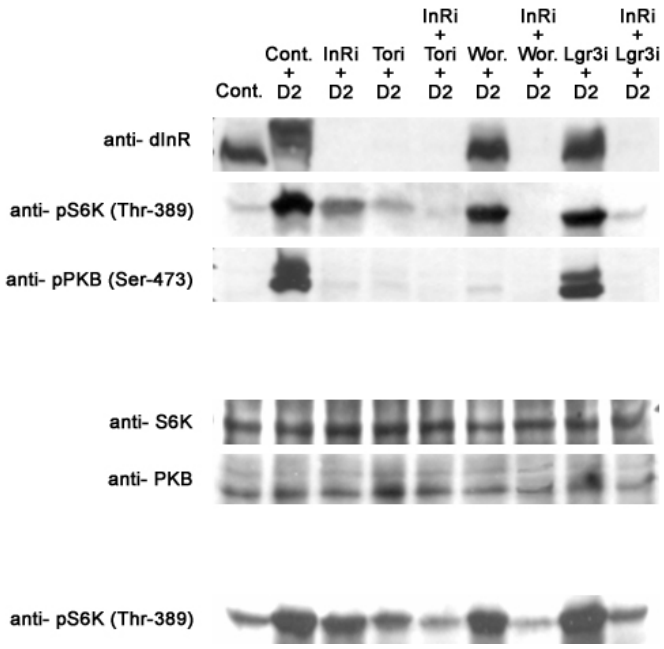


Figure 1b shows phosphorylation of S6 kinase and dPKB upon stimulation of Kc cells, which were treated either with dsRNA for dInR (InRi), dTor (Tori), dLgr3 (Lgr3i) or Wortmannin (Wort.) and/or DILP 2 (D2). The slight phosphorylation of S6 kinase in Lane 3 is not present when dTor or dLgr3 are downregulated. Anti- dInR shows robust downregulation of dInR. Treatment with Wortmannin abolishes phosphorylation of dPKB but not dS6 kinase. Antibodies against total S6 kinase and total PKB show equal proteins were loaded.

Hence, I down regulated the catalytic subunit of each member of the three classes of PI3 kinases present in Drosophila, along with dInR. To confirm that dLGR3 was having any influence on S6 kinase, when stimulated with DILP 2, I used dsRNA against a different region of dLgr3. To test whether a GPCR is really involved, Forskolin could be used to mimic the effects of activation of a GPCR. Forskolin can increase the amount of cAMP in cells, thus mimicking the effects of stimulation of a GPCR [7] (Fig 1c).

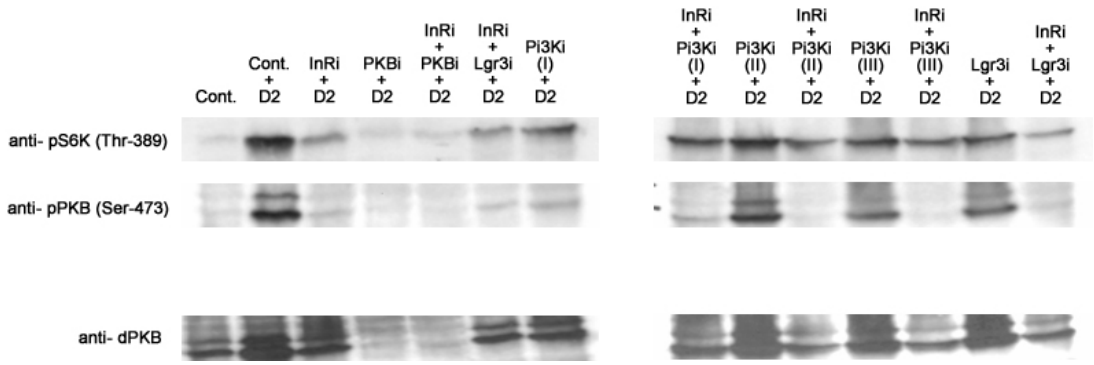


Figure 1c shows phosphorylation of dS6 kinase and dPKB upon stimulation of Kc cells, which were treated either with dsRNA for dInR (InRi), dPkb (PKBi), dLgr3 (Lgr3i), Pi3K Class I [Pi3Ki (I)], Pi3K Class II [Pi3Ki (II)], Pi3K Class III [Pi3Ki (III)] and/or DILP 2 (D2). The slight phosphorylation of S6 kinase in Lane 3 is not present when dPkb is downregulated. The slight activation of dS6 kinase is not abolished when any of the members of PI3K Classes were down regulated. Although dPKB phosphorylation was downregulated upon knockdown of Class I Pi3K but not by downregulating members of other classes. Last two lanes show that the dsRNA directed against a new region of dLgr3 does not affect the phosphorylation of S6 kinase.

To assess the role of dPKB in S6 kinase activation, I down regulated dPKB along with dInR. Fig 1c shows that indeed dPKB was getting activated even when dInR was downregulated, since downregulation of dPKB along with dInR did not result in S6 kinase activation anymore. This could be because dInR is not knocked-out, it is only 'knocked down', which means that there could be some residual receptor translated, which could not be detected by the anti-dInr antibody. Similarly the sensitivity of anti phospho-PKB antibody could be lower than that of the anti phospho-S6 kinase antibody and therefore slight phosphorylation of PKB could not be detected. This could partly be the case as is shown in Fig 1e.

Targeting a different sequence of dLgr3 did not reproduce the results as seen previously, and the use of Forskolin did not have any effect on S6 Kinase activity (Fig 1d). Taken together, these findings suggest that dInR is the sole receptor signaling to S6 Kinase via activation of PKB in response to DILP 2.

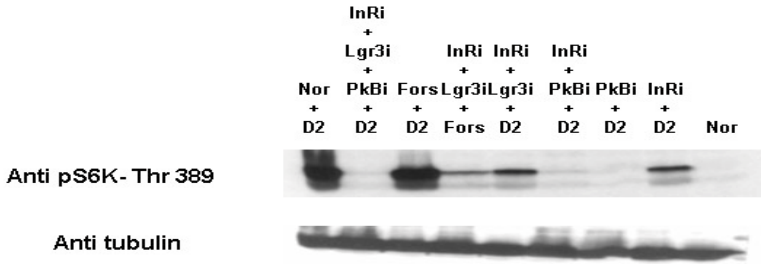


Figure 1d shows that the slight phosphorylation of S6 kinase cannot be mimicked by Forskolin stimulation. Lanes 7 and 8 (from left), confirm that the extra phosphorylation is abolished when dPKB is also downregulated under these conditions.

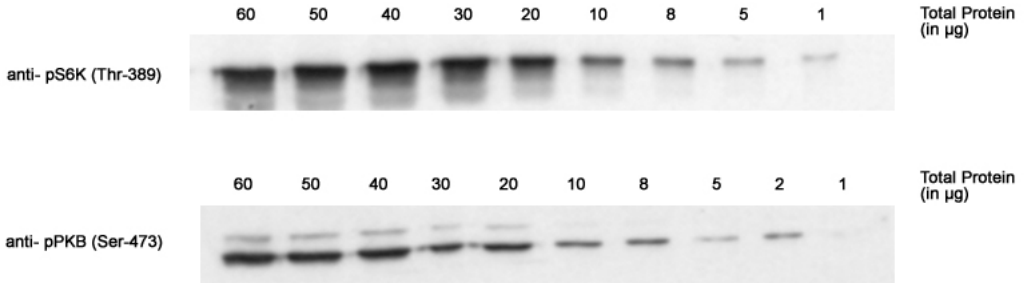


Figure 1e shows, the lowest amount of phosphorylated protein detectable by anti- pS6 kinase and anti- pPKB antibody. The blots show that anti- pS6 kinase antibody is more sensitive than anti- pPKB antibody.

Another observation which came up during these experiments was that the use of PD 098059 enhanced the phosphorylation of S6 Kinase. PD 098059 is a known inhibitor of MAPKK or MEK1/2 [8]. This implied a negative effect of MEK1/2 on S6 Kinase phosphorylation, but till now only positive effects of Ras-MAPK have been reported on PKB-PI3K pathway, like Rodriguez-Viciana et al. [9] showed that PI3 kinase is a direct target of Ras. Ma et al [2] showed that ERK 1/2 can directly inactivate the TSC 1/2 complex. Both these papers suggest a positive relationship between the two pathways. But the results I obtained suggested a negative effect of ERK1/2-MEK on S6 kinase phosphorylation. To test whether MEK really has any effect on S6 Kinase phosphorylation, I used another inhibitor, U0126, to see if it can mimic the effects of PD 098059. Furthermore, I targeted dMek by RNAi to see if this also reproduces the effect of PD. Both these experiments could not reproduce the original observation seen by PD 098059 (Fig. 2a). Hence, PD 098059 in this case is not necessarily inhibiting only MEK.

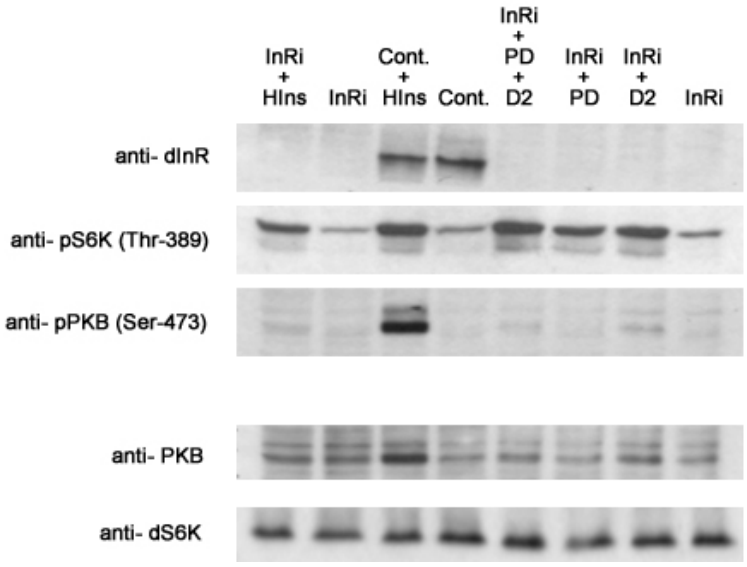
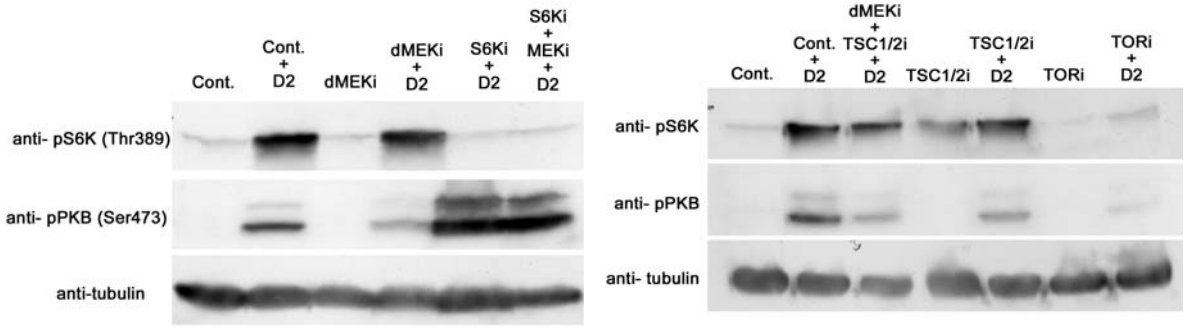


Figure 2a, Lanes 1-4 show the confirmation of the initial observation when using Human insulin instead of DILP 2. The last four lanes show the use of PD 098059 where phosphorylation of S6 kinase is slightly enhanced by use of PD.

In the same experiment where I down regulated dMek by RNAi, I observed that S6K phosphorylation remained normal, but dPKB phosphorylation goes down (Fig 2b, below). Ma et. al. have shown that ERK1/2 in mammalian system can phosphorylate TSC2 thereby disrupting the TSC1/2 complex formation. TSC2 is also known to be directly phosphorylated by PKB in Drosophila system. However, the serines and threonines phosphorylated by both these kinases (PKB and ERK1/2) are different; PKB phosphorylates Ser-939 and ERK1/2 phosphorylates Ser-664 and Ser-540.



Taken together, it can be concluded that DILP 2 signals to dS6 kinase solely via the dInR and dPKB.

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Chapter 6

Tissue variation- Insulin receptor and response to Insulin

Introduction

Research of several laboratories around the world has focused on studying how different tissues respond to insulin. A wealth of information is available on the subject, and it is beyond the scope of this chapter to touch all the aspects of tissue response to Insulin.

Most of the knowledge today on how various cell types or tissues respond to Insulin stimulation has come from studies in mammalian systems. Such studies have been done using various tissues in mice or on cell lines derived from various tissues. Fat or adipose tissue, skeletal muscles and hepatic tissues have emerged as classical insulin responsive tissues in mammals. Insulin response cannot be studied in isolation since the response is regulated by many other cell extrinsic and intrinsic factors. Studies have shown that the responses to insulin of tissues like fat, skeletal or hepatic differ significantly. Although these tissues receive the insulin signal through the insulin receptor, the downstream events are quite unique. The differentiated cells express different sets of downstream effectors which can be stimulated by the insulin receptor. There could also be variability at the receptor level. For example, the receptor could exist in tissue-specific isoforms or the amount of receptor expressed in a particular tissue types can vary at different stages or under various conditions.

Indeed the mammalian Insulin receptor can exist as two splice isoforms. The insulin receptor (IR) is expressed as two mRNA species derived from alternative splicing of exon 11 of the IR gene. Exon 11 consists of 36 nucleotides, which encode 12 amino acids at the C-terminus of the alpha-subunit [1]. The relative abundance of the mRNAs encoding isotypes A (exon 11-) and B (exon 11+) of the IR is regulated in a tissue-specific manner in both humans and rats [2, 3]. It is also regulated by the stage of development and by cell differentiation, with IR-A being the predominant IR isoform in fetal tissues and cancer cells [4]. The two IRs have been reported to exhibit distinct functional properties. IR-A shows a higher affinity for insulin and a higher internalization rate than IR-B [5, 6] whereas IR-B is considered to transmit the insulin signal more efficiently than IR-A since it has a greater kinase activity [7, 8]. Furthermore, recent studies have shown that IR-A and IR-B activate different downstream pathways. In pancreatic beta-cells, IR-A regulates insulin gene expression and IR-B does the same with beta-glucokinase but using different classes of phosphatidylinositol 3-kinase

(PI3K) [9]. In 32D cells, a murine hematopoietic cell line, IR-A sends mitogenic, antiapoptotic signals in response to insulin-like growth factor-II, whereas IR-B is more effective in inducing differentiation [10]. An altered relative expression of IR isoforms may contribute to the development of insulin resistance. In this regard, it has been demonstrated that a deregulated IR alternative splicing, resulting in a switch to IR-A, correlates with insulin resistance in myotonic dystrophy type I [11].

Serrano et al. [12] have shown that differential gene expression of IRs and IRSs in several rat target tissues of insulin action. There is a specific decrease in the expression of the IR-B isoform, as well as both mRNA and protein levels of IR, IRS-1 and IRS-3 being significantly decreased, in epididymal adipose tissue from old compared with young adult rats. They concluded that the down-regulation of early components of the insulin signal transduction pathway in a primary insulin target tissue could be related to the insulin resistance of aging.

Such studies have not picked up momentum in lower organisms. Although an insulin receptor homolog has been identified in C. elegans and Drosophila [13], detailed studies on the signaling characteristics of the receptors are still lacking. The identification of seven insulin-like ligands in Drosophila has rendered these analyses even more important.

The structure and processing of the Drosophila insulin proreceptor is somewhat different from those of the mammalian Insulin and IGF 1 receptor precursors. The InR proreceptor [MW 280 kDa] is processed proteolytically to generate an insulin-binding alpha subunit [MW 120 kDa] and a beta subunit [MW 170 kDa] with protein tyrosine kinase domain. The InR beta 170 kDa subunit contains a novel domain at the carboxyterminal side of the tyrosine kinase, in the form of a 60 kDa extension that contains multiple potential tyrosine autophosphorylation sites. This 60 kDa C-terminal domain undergoes cell-specific proteolytic cleavage that leads to the generation of a total of four polypeptides (α 120, β170, β 90, and a free 60 kDa C-terminus) from the dinr gene. These subunits assemble into mature InR receptors with the structures $\alpha_2(\beta\text{-}170)_2$ or $\alpha_2(\beta\text{-}190)_2$ [13].

The 400-amino acid, carboxyl-terminal extension contains 9 tyrosine residues, four of which are present in YXXM or YXXL motifs, suggesting that they function as binding sites for SH2 domain-containing signaling proteins. The presence of multiple putative SH2 domain binding sites in the InR represents a significant difference from its mammalian homologs and suggests that, unlike the human insulin and Insulin-like growth factor 1 receptors, the InR forms stable complexes with signaling molecules as part of its signal transduction mechanism [14].

Since this splicing variation of the Drosophila insulin receptor mRNA seems to be cell specific [13], I wanted to study the abundance of the two isoforms of the Drosophila insulin receptor in various larval tissues. I used anti-dInR (antibody kindly provided by R. Fernandez) which is directed against the β -subunit of the insulin receptor. The epitope against which the antibody is directed lies on the C-terminal extension of the dInR, which means the antibody can recognize 170kD (total β subunit pool, represents roughly the untruncated receptor form, Isoform I) and 60 kDa separated extension (represents roughly the truncated receptor form, Isoform II).

The following experiments were attempted to study how abundance, expression pattern and processing of the Drosophila Insulin receptor varies in different larval tissues. I also tested whether tissues differ in the phosphorylation of downstream kinases upon DILP 2 stimulation. I also wanted to test how low nutrient conditions would change the response of tissues to DILP stimulation. Various Drosophila cells lines, namely S2, Kc and Clone 8 cells were tested for their response to stimulation with various ligands such as human insulin, bovine insulin and IGF 1.

Materials and Method

dInr expression in various larval tissues

Fat body, gut, brain and muscles were collected from ~20 wandering third instar larvae. The tissues were collected in 2x SDS loading buffer, homogenized and heated to 95 °C for 10 minutes. The lysate was centrifuged and supernatant collected. Protein concentration were estimated using standards suspended in loading buffer. Equal amounts of total protein was loaded on an SDS-PAGE gel and blotted on a nitrocellulose membrane. The membrane was treated with anti-dInR, anti-pPKB, anti-pS6K and anti-tubulin antibodies.

Tissue collection and DILP 2 stimulation

Tissues like the brain, muscles, gut and salivary glands from normally fed or starved larvae (~20 in number) dissected and collected separately in serum free Schneider's medium. After one hour of incubation in serum free medium, DILP 2 was added to a final concentration of 100nM. After incubation for 20 minutes, the tissues were centrifuged at 300xg at 4°C for 10min and the supernatant was removed. The tissues were washed twice with ice-cold PBS and lysed with lysis buffer for one hour. The tissues were crushed with a pestle and 25µl of the lysate was used directly for protein estimation. Equal amount of total protein (20-50µg)

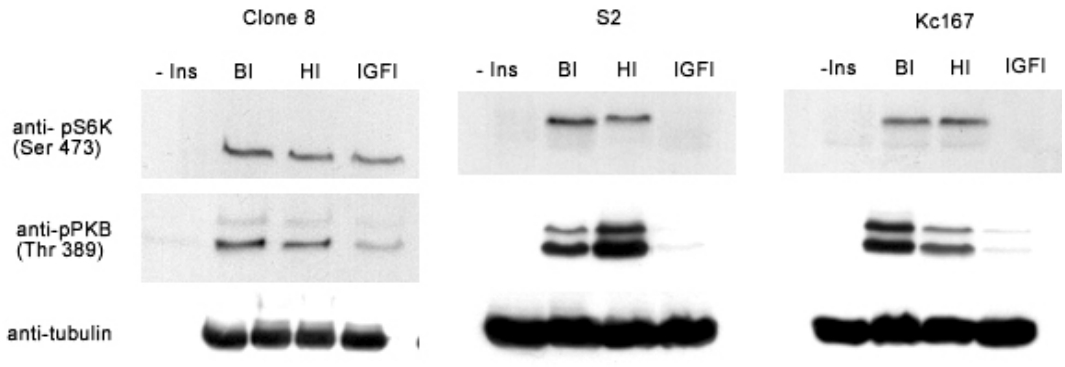
was loaded onto a 10% SDS-PAGE gel and western blotting was done. Rest of the procedure is the same as described in previous chapters. For starvation second instar larvae were picked and fed on 10% Sucrose containing agarose for 24 hours. The larvae were collected, dissected and extracts were prepared as described above.

Stimulation by various ligands in cell culture

S2, Kc and Clone 8 cells were grown to 80-90% confluence in six-well plates and stimulated with 100nM bovine insulin, human insulin or IGF 1 for 20 minutes. The cells were collected and protein was extracted as described in previous chapters. The lysates were tested for phosphorylation of S6 kinase and PKB and for tubulin.

Results and discussion

Fig 1a compares the phosphorylation of PKB and S6 kinase in Clone 8, S2 and Kc cells in response to bovine insulin, human insulin and IGF 1. Bovine and human insulin were comparable in their potency on Kc and S2 cells. IGF 1 evoked a much weaker phosphorylation. Clone 8 cells showed a weaker response to any of the factors, as compared to S2 and Kc cells. Indeed, in one of the later experiments, it was shown that dInR is weakly expressed in these cells as compared to S2 and Kc cells (data not shown). These results show that the Drosophila insulin receptor is more similar to the human Insulin receptor than the human IGF 1 receptor, hence it binds more efficiently to molecules structurally more similar to Insulin than to IGFs.



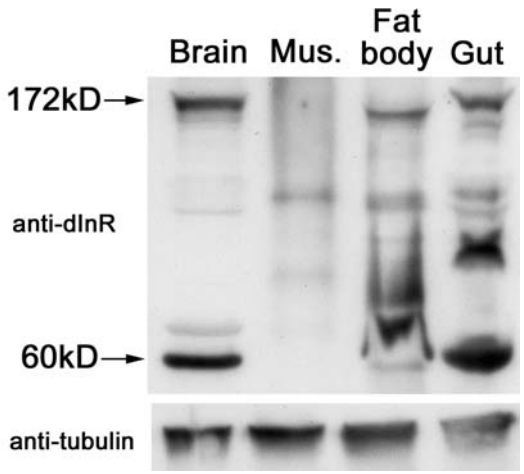


Figure 1b shows expression of β-subunit of the dInR in larval brain, muscles, fat body and gut, using an antibody directed against the 60kDa C-terminal extension of the subunit. The larval brain and gut show expression of the untruncated ~172kDa β-subunit (Isoform I) and separate 60kDa C-terminal extension (Isoform II). Note that the quantities of both the untruncated and the extension are roughly equal in brain, whereas gut shows more truncated form as compared to the untruncated form. The fat body shows overall less expression of the untruncated β-subunit (Isoform I) and very little expression of the C-terminal extension (Isoform II). Surprisingly, larval muscles do not show any expression of the β-subunit of the dInR. Hence, various larval tissues differ in the expression of the two isoforms of dInR.

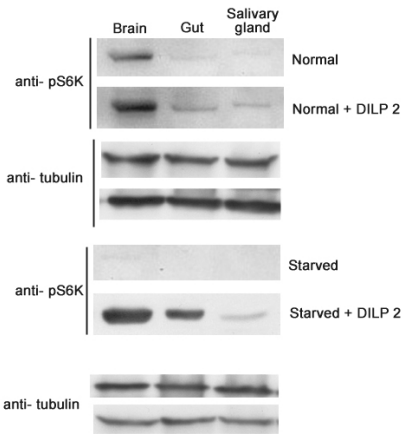


Figure 1c shows phosphorylation of S6 kinase upon DILP 2 stimulation in three different larval tissues- brain, gut and salivary gland. Under normal feeding conditions, S6 kinase phosphorylation was high in larval brain and almost undetectable in gut and salivary glands. Upon stimulation with DILP 2, S6 kinase phosphorylation increased slightly in the brain, although the signal in gut and salivary glands was still low. The difference in the intensities of the signal could be due to differential expression of the Insulin receptor in these tissues (Figure 1b).

Upon starvation of larvae, the S6 kinase signal in the brain decreased significantly, suggesting a lowering of general DILP levels, when nutrient availability is low. When such tissues were stimulated with DILP 2 in Schneider's medium, the signal of S6 kinase phosphorylation was

much higher as compared to stimulation under non-starved conditions. This could indicate that under starvation conditions (low nutrient availability) the cells become ‘sensitized’ to insulin signaling probably by expressing more Insulin receptor or some other pathway component. Further experiments would include comparison of the amount of InR expressed in various tissues under normal and restricted nutrient conditions.

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Conclusion and Outlook

As the components of the Insulin/IGF signaling pathway were discovered and characterized, it became clear that the pathway plays a pivotal role in fundamental life processes like growth and metabolism. It also became evident that aberrations in the functioning of the pathway components could lead to serious disease conditions like Diabetes and Cancer. Besides studies on human patients and in cell culture, mouse as a model organism has added indispensable knowledge to our understanding of the Insulin signaling system. The Insulin/IGF signaling pathway is strikingly well conserved in lower model organisms like *Drosophila melanogaster* and *Caenorhabditis elegans*. Experiments using Drosophila to identify and characterize new insulin pathway components have been done by many labs. However, the insulin-like ligands themselves are less well characterized in lower model organisms.

This Ph.D. project aimed at the biochemical characterization of the seven putative Drosophila Insulin-like peptides (DILPs). A combination of genetic, molecular and biochemical techniques were employed in characterizing the DILPs. To have four of the DILPs (namely DILP 2, DILP 3, DILP 4 and DILP 5) purified, helped significantly in deciphering the similarities and differences between the actions of the various DILPs. Genetics and cell culture studies suggest that all of the seven Drosophila Insulins are positive regulators of growth. The growth promoting potentials of the DILPs, both in cell culture and in *in vivo* assays showed variation, suggesting that the DILPs differ in their signaling potentials. DILP 2 emerged as the strongest activator in all the assays tested. It was not possible to characterize the role of DILPs on sugar/glucose uptake in the assays designed and performed in cell culture and on tissues. This does not rule out the possibility that DILPs play a role in sugar homeostasis in Drosophila. Study of the expression pattern of the single Drosophila insulin receptor showed that various Drosophila tissues differ in the amount and isoform-specific expression. Lastly experiments showed that lower nutrient availability renders the Drosophila tissues more ‘sensitive’ to insulin signaling. This project, one of the few done on studying insulin-like ligands in lower model organisms, has shed some light on the roles that the unusually high number of insulin-like molecules or genes might be playing in lower organisms. Data on DILPs suggest that these insulin-like peptides might have redundant functions in lower organisms. Many of these might be binding and signaling through the same classical insulin receptor but just vary in their strengths. The physiological importance of the variations in the signaling intensities of similar ligands towards a single receptor is unclear. It is possible that the growth and metabolism regulating functions of these peptides are not

evenly distributed among these ligands. This means that possibly the main job is performed by one or two of the peptides and the rest are either not expressed or not secreted at all. Perhaps, due to this reason the number of such peptides and genes decreased during evolution, thus getting rid of the redundant insulin-like genes. However, this does not negate the possibility that some of these ligands in lower organisms actually bind to completely different receptors and regulate completely different physiological processes.

Although this study has given important clues on the functioning of the DILPs, it is far from exhaustive. There are still many questions to be answered regarding the Dilps. The biggest advantage of Drosophila as a model organism is the ease of genetic manipulation. There are a variety of genetic approaches possible in Drosophila for manipulation of desired genes. Creating loss-of-function mutants for the *dilps* is of prime importance as it will help in clearly establishing their physiological functions. Up to date mutants for *dilps* have not yet been reported from random mutagenesis screens (forward genetics) performed in several laboratories. This could, to a certain extent be due to the redundancy of *dilp* functions. To overcome the problem of redundancy, creating double or triple mutants for the *dilps* might be required. There are several comprehensive reverse genetic approaches that could be employed in generating *dilp* mutants. Several projects in laboratories around the world are aiming to create P-element (transposable elements) insertions in practically every gene in the Drosophila genome. If lines carrying P-elements or other insertions (like Piggybacs) in *dilp* genes become available, they themselves will serve as mutants or the P-elements can be easily used to create chromosomal deletions in one or more of the *dilps*. Gene targeting is also possible via homologus recombination by using 'ends-in' (insertional gene targeting) or 'ends-out' (replacement gene targeting) techniques. Lastly, knocking down genes using RNA interference (RNAi) has gained importance in the recent years. Attempts to drive RNAi against *dilps* in Dilp producing neurons have not been successful so far, probably because of neuronal tissues being resistant to this phenomenon. However, in the future, with further development of this technique, it could be possible to use RNAi to knockdown *dilp* function.

One of the important aspects of insulin- or diabetes-study in mammals has been the regulation of insulin expression and secretion. Advances have been made in this direction in mammals but very little is known about their regulation in lower organisms (e.g. for the Dilps). It is possible that the regulation of the Dilps could be occurring at more than one level, e.g. at the level of expression of RNA and protein, and at the level of DILP secretion. Further studies should be focusing on the regulation of the Dilps since if these mechanisms are similar to that working for human insulin, then Drosophila can become a very useful model for Diabetes. On the other hand, genetic approaches in Drosophila can help to study what signaling pathways

and events might be working to regulate the RNA and protein expression of the Dilps. There could be another level of regulation of the DILPs at their transport level. Mammalian data clearly show that binding partners, during circulation of factors like IGFs, play a very important role in regulating their action. The binding proteins influence the stability and receptor interaction of these factors. In one of the genetic screens in our lab, one such gene named ImpL2 was identified. Genetic and biochemical analyses of ImpL2 suggest that it could be binding to at least one of the DILPs. How binding of such proteins might affect DILP stability and activity remains to be studied. It is possible that there are more binding partners for the DILPs encoded by the Drosophila genome.

In mammals, insulin regulates homeostasis in the body by acting in synergy with several other hormonal factors. The most important of these factors is the hormone Glucagon, which exerts opposite effects than Insulin. The balancing actions of these two hormones maintain the glucose levels in blood to a constant level. In insects, Adipokinetic hormone (AKH) is considered to be a functional homolog of Glucagon. Recent studies focusing on the characterization of Akh have suggested that Akh might indeed work with Dilps to maintain the haemolymph sugar homeostasis in Drosophila. Detailed studies on AKH and DILP action would show whether the mechanism of maintenance of sugar homeostasis in Drosophila is similar to that in humans.

Drosophila melanogaster is a popular model organism mainly because of the strength of Drosophila genetics. It has now been realized that the small fruitfly, which separated with humans several million years ago during evolution, can actually prove to be very useful as a model system for many threatening diseases of these times, like Cancer, Diabetes, Alzheimer's or Parkinson's. It is obvious that Drosophila can contribute significantly not only to understanding mechanisms of such diseases but also to finding drugs to fight these diseases.

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